3 Membranes and Cell Organelles

3.1 The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol.
Graham, J., Ford, T. and Rickwood, D.
*Anal. Biochem., **220**, 367-373 (1994*)

This paper reports the use of a new density gradient compound, **iodixanol**, for the resolution of the major organelles from mouse liver. A major advantage of iodixanol over other iodinated density gradient media is its ready ability to form self-generated gradients. Gradient-forming conditions have been modulated to provide optimal recoveries of Golgi membranes, lysosomes, mitochondria and peroxisomes. The organelles were isolated in high yield (80-90% of gradient input) and high purity. Nycodenz and iodixanol were compared using preformed gradients. Iodixanol provided resolution superior to that of Nycodenz, notably of peroxisomes and mitochondria and the separation of lysosomes from endoplasmic reticulum. Because iodixanol does not interfere significantly with marker enzyme activities, gradient fractions can be analyzed without removal of the gradient media.

3.2 A detergent-free method for purifying caveolae membrane from tissue culture cells.
Smart, E.J., Ying, Y-S, Mineo, C. and Anderson, R.G.W.
*Proc. Natl. Acad. Sci. USA, **92**, 10104-10108 (1995)*

Current methods for purifying caveolae from tissue culture cells take advantage of the Triton X-100 insolubility of this membrane domain. To circumvent the use of detergents, we have developed a method that depends upon the unique buoyant density of caveolae membrane. The caveolae fractions that we obtain are highly enriched in caveolin. As a consequence we are able to identify caveolae-associated proteins that had previously gone undetected. Moreover, resident caveolae proteins that are soluble in Triton X-100 are retained during the isolation.

3.3 Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae.
Shaul, P.W. et al
*J. Biol. Chem., **271(11)**, 6518-6522 (1996)*

Endothelial nitric-oxide synthase (eNOS) generates the key signaling molecule nitric oxide in response to intralumenal hormonal and mechanical stimuli. We designed studies to determine whether eNOS is localized to plasmalemmal microdomains implicated in signal transduction called caveolae. Using immunoblot analysis, eNOS protein was detected in caveolar membrane fractions isolated from endothelial cell plasma membranes by a newly developed detergent-free method; eNOS protein was not found in noncaveolar plasma membrane. Similarly, NOS enzymatic activity was 9.4-fold enriched in caveolar membrane versus whole plasma membrane, whereas it was undetectable in non-caveolar plasma membrane. 51-86% of total NOS activity in postnuclear supernatant was recovered in plasma membrane, and 57-100% of activity in plasma membrane was recovered in caveolae. Immunoelectron microscopy showed that eNOS heavily decorated endothelial caveolae, whereas coated pits and smooth plasma membrane were devoid of gold particles. Furthermore, eNOS was targeted to caveolae in COS-7 cells transfected with wild-type eNOS cDNA. Studies with eNOS mutants revealed that both myristoylation and palmitoylation are required to target the enzyme to caveolae and that each acylation process enhances targeting by 10-fold. Thus, acylation targets eNOS to plasmalemmal caveolae. Localization to this microdomain is likely to optimize eNOS activation and the extracellular release of nitric oxide.

3.4 Clustered folate receptors deliver 5-methyltetrahydrofolate to cytoplasm of MA104 cells
Smart, E.J., Mineo, C. and Anderson, R.G.W.
*J. Cell Biol., **134(5)**, 1169-1177 (1996)*

Previously, a high affinity, glycosylphosphatidylinositol-anchored receptor for folate and a caveolae internalization cycle have been found necessary for potocytosis of 5-methyltetrahydrofolate in MA104. We now show by cell fractionation that folate receptors also must be clustered in caveolae for potocytosis. An enriched fraction of caveolae from control cells retained 65-70% of the [^{3}H]folic acid bound to cells in culture. Exposure of cells to the cholesterol-binding drug, filipin, which is known to uncluster receptors, shifted approximately 50% of the bound [^{3}H]folic acid from the caveolae fraction to the noncaveolae membrane fraction and markedly inhibited internalization of [^{3}H]folic acid. An mAb directed against the folate receptor also shifted approximately 50% of the caveolae associated [^{3}H]folic acid to noncaveolae membrane, indicating the antibody perturbs the normal receptor distribution. Concordantly, the mAb

inhibited the delivery of 5-methyl[^{3}H]tetrahydrofolate to the cytoplasm. Receptor bound 5-methyl [^{3}H]tetrahydrofolate moved directly from caveolae to the cytoplasm and was not blocked by phenylarsine oxide, an inhibitor of receptor-mediated endocytosis. These results suggest cell fractionation can be used to study the uptake of molecules by caveolae.

3.5 Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane.
Mineo, C., James, G.L., Smart, E.J. and Anderson, R.G.W.
*J. Biol. Chem., **271(20)**, 11930-11935 (1996)*

An essential step in the epidermal growth factor (EGF)-dependent activation of MAP kinase is the recruitment of Raf-1 to the plasma membrane. Here we present evidence that caveolae are the membrane site where Raf-1 is recruited. Caveolae fractions prepared from normal Rat-1 cells grown in the absence of serum were highly enriched in both EGF receptors and Ras. Thirty seconds after EGF was added to these cells Raf-1 began to appear in caveolae but not in non-caveolae membrane fractions. The maximum concentration was reached at 3 min followed by a decline over the next 60 min. During this time EGF receptors disappeared from the caveolae fraction while the concentration of Ras remained constant. The Raf-1 in this fraction was able to phosphorylate MAP kinase, whereas cytoplasmic Raf-1 in the same cell was inactive. Elevation of cellular cAMP blocked the recruitment of Raf-1 to caveolae. Overexpression of Ha-Ras^{v12} caused the recruitment of Raf-1 to caveolae independently of EGF stimulation, and this was blocked by the farnesyltransferase inhibitor BZA-5B. Finally, prenylation appeared to be required for localization of Ras to caveolae.

3.6 A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane.
Smart, E.J., Ying, Ys., Donzell, W.C. and Anderson, R.G.W.
*J. Biol. Chem., **271(46)**, 29427-29435 (1996)*

Caveolin is a 22-kDa membrane protein found associated with a coat material decorating the inner membrane surface of caveolae. A remarkable feature of this protein is its ability to migrate from caveolae directly to the endoplasmic reticulum (ER) when membrane cholesterol is oxidized. We now present evidence caveolin is involved in transporting newly synthesized cholesterol from the ER directly to caveolae. MA104 cells and normal human fibroblasts transported new cholesterol to caveolae with a half-time of approximately 10 min. The cholesterol then rapidly flowed from caveolae to non-caveolae membrane. Cholesterol moved out of caveolae even when the supply of fresh cholesterol from the ER was interrupted. Treatment of cells with 10 μg/ml progesterone blocked cholesterol movement from ER to caveolae. Simultaneously, caveolin accumulated in the lumen of the ER, suggesting cholesterol transport is linked to caveolin movement. Caveolae fractions from cells expressing caveolin were enriched in cholesterol 3-4-fold, while the same fractions from cells lacking caveolin were not enriched. Cholesterol transport to the cell surface was nearly 4 times more rapid in cells expressing caveolin than in matched cells lacking caveolin.

3.7 Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains.
Vey, M. et al
*Proc. Natl. Acad. Sci. USA, **93**, 14945-14949 (1996)*

Results of transgenetic studies argue that the scrapie isoform of the prion protein (PrP^{Sc}) interacts with the substrate cellular PrP (PrP^{C}) during conversion into nascent PrP^{Sc}. While PrP^{Sc} appears to accumulate primarily in lysosomes, caveolae-like domains (CLDs) have been suggested to be the site where PrP^{C} is converted into PrP^{Sc}. We report herein that CLDs isolated from scrapie-infected neuroblastoma (ScN2a) cells contain PrP^{C} and PrP^{Sc}. After lysis of ScN2a cells in ice-cold Triton X-100, both PrP isoforms and an N-terminally truncated form of PrP^{C} (PrP^{C}-II) were found concentrated in detergent-insoluble complexes resembling CLDs that were isolated by flotation in sucrose gradients. Similar results were obtained when CLDs were purified from plasma membranes by sonication and gradient centrifugation; with this procedure no detergents are used, which minimizes artifacts that might arise from redistribution of proteins among subcellular fractions. The caveolar markers ganglioside GM1 and H-ras were found concentrated in the CLD fractions. When plasma membrane proteins were labeled with the impermeant reagent sulfo-N-hydroxysuccinimide-biotin, both PrP^{C} and PrP^{Sc} were found biotinylated in CLD fractions. Similar results on the colocalization of PrP^{C} and PrP^{Sc} were obtained when CLDs were isolated from Syrian hamster brains. Our findings demonstrate that both PrP^{C} and PrP^{Sc} are present in CLDs and, thus, support the hypothesis that the PrP^{Sc} formation occurs within this subcellular compartment.

3.8 Iodixanol (OptiPrep), an improved density gradient medium for the iso-osmotic isolation of rat liver peroxisomes.
Van Veldhoven, P.P., Baumgart, E. and Mannaerts, G.P.
Anal. Biochem. ***237****, 17-23 (1996)*

The suitability of **iodixanol**, a nonionic iodinated compound with a molecular weight of 1550, for the isolation of peroxisomes from rat liver was investigated. Centrifugation of light mitochondrial fractions in 20 to 40% (w/v) iodixanol gradients, made iso-osmotic by the addition of sucrose, resulted in an excellent separation of peroxisomes from the remaining organelles, which were not able to enter the gradient. Peroxisomes banded around 30% (w/v) iodixanol (d–1.175) and, as revealed by marker enzyme analysis, were enriched 35- to 40-fold. Morphological examination of the peroxisomal fractions confirmed the near absence of other organelles and revealed structurally well-preserved peroxisomes. Free cores, also present in the starting fractions, migrated to higher densities and were trapped on a cushion. No interference of iodixanol with marker enzyme determinations was observed, except for the UV-metric determination of urate oxidase and for the analysis of protein.

3.9 Potential chitinase activating factor from yeast cells of *Candida albicans*.
Jackson, D.J., Saunders, V.A. and Humphreys, A.M.
Lett. Appl. Microbiol., ***23****, 159-162 (1996)*

Microsomal chitinase from yeast and hyphal cells of *Candida albicans* was activated endogenously by incubation at 30^0C and exogenously by trypsin. The putative activating factor of yeast cells was separated from chitinase activity by fractionation of lysed protoplasts on an **Iodixanol** density gradient. The vacuole fraction contained no significant chitinase activity, but was enriched in chitinase activating factor. Activity of microsomal chitinase increased upon incubation with this, but no other gradient factor. Results suggest that the regulatory system governing microsomal chitinase activity, like that governing chitin synthase, involves a >vacuolar= activating factor in *Candida albicans*.

3.10 Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae
Liu, P., Ying, Y., Ko, Y.G and Anderson, R.G.W.
J Biol. Chem., 271(17), 10299-10303 (1996)

Previously we showed that interleukin lβ stimulates the conversion of sphingomyelin to ceramide in the caveolae fraction of normal human fibroblasts. The ceramide, in turn, blocked platelet-derived growth factor (PDGF) stimulated DNA synthesis. We now present evidence that the PDGF receptor initiates signal transduction from caveolae. Cell fractionation and immunocytochemistry show caveolae to be the principal location of PDGF receptors at the cell surface. Multiple caveolae proteins acquire phosphotyrosine when PDGF binds to its receptor, but the hormone appears to have little effect on the tyrosine phosphorylation of non-caveolae membrane proteins. Five proteins known to interact with the phosphorylated receptor were found to be highly enriched in caveolae membrane. PDGF caused the concentration of three of these proteins to significantly increase in the caveolae fraction. Finally, PDGF stimulated the association of a 190-kDa phosphoprotein with the caveolae marker protein, caveolin. Therefore, ceramide may modulate PDGF receptor function directly in caveolae.

3.11 Tissue-specific distribution and subcellular distribution of phospholipase D in rat: evidence for distinct RhoA- and ADP-ribosylation factor (ARF)-regulated isoenzymes.
Provost, J.J. et al.
Biochem. J., ***319(2)****, 285-291 (1996)*

Phospholipase D (PLD) is regulated by many factors including the small G-proteins, RhoA and ADP-ribosylation factor (ARF). The present study examined the distribution of RhoA- and ARF-responsive PLD in membranes, microsomes and cytosol of rat tissues and in rat liver subcellular fractions. PLD was present in all tissue fractions examined and was stimulated by guanosine 5'-[γ -thio]triphosphate (GTP[S]), with the highest specific activities being in lung, kidney and spleen. When myristoylated recombinant ARF (mARF) was added with GTP[S], the PLD activity was stimulated further, but the addition of RhoA was without effect. However, in extracts from crude membranes both mARF and RhoA enhanced the stimulation by GTP[S], with high specific activities of PLD being observed in all tissues except muscle. The response to mARF was usually greater than to RhoA, and the responses were additive, except for liver, which showed synergism. When the PLD activity of subcellular fractions of liver was examined, GTP[S] caused increases in all fractions except microsomes and mitochondria, which exhibited low activity. All fractions except mitochondria showed responses to RhoA and mARF, with the response to RhoA being greater in plasma membranes and that to

mARF being greater in Golgi and nuclei. Western blotting showed that RhoA was located mainly in the cytosol and plasma membranes, whereas ARF was principally in the cytosol. These findings demonstrate the widespread occurrence of significant activity of both Rho- and ARF-responsive forms of PLD in membranes from all tissues except muscle, and the presence of both forms in liver subcellular fractions except mitochondria. The large variations in the relative responses of PLD to Rho and ARF observed in different tissues and fractions support the existence of different isoforms of the enzyme.

3.12 Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells
Majoul, I.V., Bastiaens, P.I.H. and Soling H-D.
*J. Cell Biol., **133**, 777-789 (1996)*

The A2 chain of cholera toxin (CTX) contains a COOH-terminal Lys-Asp-Glu-Leu (KDEL) sequence. We have, therefore, analyzed by immunofluorescence and by subcellular fractionation in Vero cells whether CTX can be used to demonstrate a retrograde transport of KDEL proteins from the Golgi to the ER. Immunofluorescence studies reveal that after a pulse treatment with CTX, the CTX-A and B subunits CTX-A and CTX-B) reach Golgi-like structures after 15-20 min (maximum after 30 min). Between 30 and 90 min, CTX-A (but not CTX-B) appear in the intermediate compartment and in the ER, whereas the CTX-B are translocated to the lysosomes. Subcellular fractionation studies confirm these results: after CTX uptake for 15 min, CTX-A is associated only with endosomal and Golgi compartments. After 30 min, a small amount of CTX-A appears in the ER in a trypsin-resistant form, and after 60 min, a significant amount appears. CTX-A seems to be transported mainly in its oxidized form (CTX-A1-S-S-CTX-A2) from the Golgi to the ER, where it becomes slowly reduced to form free CTX A1 and CTX-A2, as indicated by experiments in which cells were homogenized 30 and 90 rain after the onset of CTX uptake in the presence of N-ethylmaleimide. Nocodazol applied after accumulation of CTX in the Golgi inhibits the appearance of CTX-A in the ER and delays the increase of 3', 5'cAMP, indicating the participation of microtubules in the retrograde Golgi-ER transport.

3.13 Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae.
Babitt, J. et al
*J. Biol. Chem., **272(20)**, 13242-13249 (1997)*

The class B, type I scavenger receptor, SR-BI, was the first molecularly well defined cell surface high density lipoprotein (HDL) receptor to be described. It mediates transfer of lipid from HDL to cells via selective lipid uptake, a mechanism distinct from receptor-mediated endocytosis via clathrin-coated pits and vesicles. SR-BI is expressed most abundantly in steroidogenic tissues (adrenal gland, ovary), where trophic hormones coordinately regulate its expression with steroidogenesis, and in the liver, where it may participate in reverse cholesterol transport. Here we have used immunochemical methods to study the structure and subcellular localization of murine SR-BI (mSR-BI) expressed either in transfected Chinese hamster ovary cells or in murine adrenocortical Y1-BS1 cells. mSR-BI, an approximately 82-kDa glycoprotein, was initially synthesized with multiple high mannose N-linked oligosaccharide chains, and some, but not all, of these were processed to complex forms during maturation of the protein in the Golgi apparatus. Metabolic labeling with [^{3}H]palmitate and [^{3}H]myristate demonstrated that mSR-BI was fatty acylated, a property shared with CD36, another class B scavenger receptor, and other proteins that concentrate in specialized, cholesterol- and glycolipid-rich plasma membrane microdomains called caveolae. **OptiPrep** density gradient fractionation of plasma membranes established that mSR-BI copurified with caveolin-1, a constituent of caveolae; and immunofluorescence microscopy demonstrated that mSR-BI colocalized with caveolin-1 in punctate microdomains across the surface of cells and on the edges of cells. Thus, mSR-BI colocalizes with caveolae, and this raises the possibility that the unique properties of these specialized cell surface domains may play a critical role in SR-BI-mediated transfer of lipids between lipoproteins and cells.

3.14 Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes.
Cartwright, I.J. et al
*J. Lipid Res., **38**, 531-545 (1997)*

Our aims were (i) to determine which lipids co-localise with newly-synthesised apo-B in the lumen of the rough endoplasmic reticulum (RER), and thus may play a role in the stabilisation and/or translocation of this

protein: and (ii) to determine the intracellular sites of assembly of lipids into VLDL. In order to do this, we have developed a new method for the separation of ER derived microsomes on self-generated gradients of iodixanol (**OptiPrep)**.
Rabbit liver microsomes were resolved into two broad peaks, the lighter peak contained smooth vesicles, and the heavier peak contained rough vesicles. Each peak was collected in a number of fractions. A single gradient thus separates the initial events in the secretion process (RER fractions), from later events (SER fractions)**.** The microsomal fractions were separated into membranes and lumenal contents, and the mass of apo-B and VLDL lipids determined by ELISA or high performance thin layer chromatography, respectively. The biosynthetic relationships of apo-B and lipids were investigated, in timed or chase-experiments, by incubation of isolated rabbit hepatocytes with radiolabelled precursors of apo-B or lipids, followed by isolation, and analysis of the microsomal fractions. The results indicate that very small amounts of triacylglycerol, cholesterol and cholesterol ester co-localise with apo-B into the lumen of RER. The bulk of the VLDL lipids were in the lumen of the SER. However, some newly synthesised triacylglycerol, phospholipid, cholesterol and cholesterol ester were transferred to the lumen of the RER and were chased into the SER lumen. Double-labelling experiments, showed that cholesterol ester produced from newly synthesised cholesterol (labelled with [^{3}H]-mevalonate and [^{14}C]-oleate) was almost exclusively present in the RER, while cholesterol ester in the SER was labelled only with [^{14}C]-oleate. Thus, distinct intracellular lipid-pools may be involved at different stages in the assembly of VLDL.

3.15 Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line.
Orlandi, P.A.
*J. Biol. Chem., **272(7)**, 4591-4599 (1997)*

A key step in the action of cholera toxin (CT) is the reduction of its A subunit to the A_1 peptide. The latter is an ADP-ribosyltransferase, which activates the α-subunit of the stimulatory G protein of adenylyl cyclase. In this study, the enzymatic reduction of membrane-bound CT in CaCo-2 human intestinal epithelial cells was characterized. Whereas diphtheria toxin was found to be reduced by a cell surface population of protein-disulfide isomerase (PDI) and its cytotoxicity was inhibited by *p*- chloromercuri-benzenesulfonic acid, bacitracin, or anti-PDI antibodies, these inhibitors had no effect on CT reduction or activity in intact cells. In contrast, the reduction of CT *in vitro* by either postnuclear supernatants (PNS) or microsomal membranes in the presence of Triton X-100 was significantly inhibited by p-chloromercuribenzenesulfonic acid and bacitracin. Anti-PDI monoclonal antibodies likewise inhibited the *in vitro* reduction of CT and also were effective in depleting reductase activity from PNS. Since inhibition and depletion were not observed in the absence of detergent, these results suggested that the reductase activity was a soluble component localized to the lumen of microsomal vesicles and correlated with the presence of protein-disulfide isomerase. This was further confirmed by showing a corresponding depletion of reductase activity and PDI in alkali-treated microsomes. This activity was restored when purified bovine PDI was added back to alkali-treated microsomes in a redox buffer that reflected conditions found in the lumen of endoplasmic reticulum (ER). When the CT-related reductase activity was assayed in subcellular fractions of PNS-derived membranes isolated on a 9-30% **iodixanol** gradient, the activity, as measured by CT-A_1 peptide formation localized to those membrane fractions containing the majority of cellular PDI. Furthermore, the banding density corresponded to a region of the gradient containing ER-derived membranes. These results indicated that CT was a substrate for PDI-catalyzed reduction in intact cells and supported the hypothesis that CT reduction and activation occurs in the ER.

3.16 The transmembrane domain of a carboxyl-terminal anchored protein determines localization to the endoplasmic reticulum.
Yang, M., Ellenberg, J., Bonifacino, J.S. and Weissman, A.M.
*J. Biol.Chem., **272(3)**, 1970-1975 (1997)*

UBC6 is a C-terminal membrane-anchored (type IV) protein, native to *Saccharomyces cerevisiae*, where it is found in the endoplasmic reticulum. When expressed in mammalian cells, this novel ubiquitin-conjugating enzyme also localizes to the endoplasmic reticulum. UBC6 lacks a lumenal domain and contains no known endoplasmic reticulum retention signals. Analysis of chimeric proteins in which the cytosolic domain of UBC is linked to a heterologous transmembrane domain, or in which the UBC6 transmembrane domain is appended to an unrelated soluble protein, led to the determination that the transmembrane domain of UBC6 plays a dominant role in its compartmental localization. The basis for the transmembrane domain-mediated subcellular targeting of UBC6 was evaluated by lengthening the wild type UBC6 hydrophobic segment from 17 to 21 amino acids, which resulted in re-targeting to the Golgi complex. A further increase in length to 26 amino

acids allowed this modified protein to traverse the secretory pathway and gain expression at the plasma membrane. These findings are consistent with models in which, in the absence of dominant cytosolic or lumenal targeting determinants, proteins may be sorted within the secretory pathway based on interactions between their transmembrane domains and the surrounding lipid bilayer.

3.17 Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane
Wu, C., Butz, S., Ying, Y-S and Anderson, R.G.W.
J. Biol. Chem., ***272(6)***, *3554-3559 (1997)*

Recent evidence suggests that tyrosine kinases are highly organized in caveolae of tissue culture cells. We now report the isolation of a membrane domain from neuronal plasma membranes that has the biochemical characteristics of caveolae. A low density membrane (LDM) fraction with the same density as caveolae was highly enriched in tyrosine kinases such as insulin receptors, neurotrophin receptors, Eph family receptors, and Fyn. Grb2, Ras, heterotrimeric GTP-binding proteins, and Erk2 were also concentrated in the LDM. Incubation of the LDM fraction at 37°C stimulated the phosphorylation on tyrosine of multiple, resident proteins, whereas the bulk membrane fraction was devoid of tyrosine kinase activity. The LDM, which makes up ~5-10% of the plasma membrane protein, appears to be organized for signal transduction.

3.18 Physical association with Ras enhances activation of membrane-bound Raf (RafCAAX)
Mineo, C., Anderson, R.G.W. and White, M.A.
J. Biol. Chem., ***272(16)***, *10345-10348 (1997)*

The transforming activity of artificially membrane targeted Rafl suggests that Ras-mediated recruitment of Rafl to the plasma membrane is an important step in Rafl activation. Cellular Ras is concentrated in the caveolae, a microdomain of the plasma membrane that is highly enriched in caveolin, glycosylphosphatidylinositol-anchored proteins, and signal transduction molecules. Growth factor stimulation recruits Rafl to this membrane domain. Whether Ras simply promotes Rafl association with caveolae membranes or also modulates subsequent activation events is presently unclear. We have identified a ras variant, ras12V,37G, that does not interact with Raf1 but does interact with a mutant raf1, raf1(257L). To examine the role of Ras in the activation of membrane-bound Raf1, raf1CAAX, and raf1(257L)CAAX membrane-targeted variants of Raf1 and raf1(257L), respectively, were expressed in fibroblasts with or without coexpression of ras12V,37G. Cell fractionation localized both raf1CAAX and raf1(257L)CAAX to caveolae membranes independent of rasl2V,37G expression; however, coexpression of rasl2V,37G enhanced the activation of raf(257L)CAAX, but not raf1CAAX, as monitored by induction of cellular transformation, increased Raf kinase activity, and induction of activated MAP kinase. These results suggest that the Ras/Raf1 interaction plays a role in Raf1 activation that is distinct from membrane recruitment.

3.19 Organization of G proteins and adenylyl cyclase at the plasma membrane
Huang, C., Hepler, J.R., Chen, L.T., Gilman, A.G., Anderson, R.G.W. and Mumby, S.M.
Mol. Biol. Cell, ***8****, 2365-2378 (1997)*

There is mounting evidence for the organization and compartmentation of signaling molecules at the plasma membrane. We find that hormone-sensitive adenylyl cyclase activity is enriched in a subset of regulatory G protein-containing fractions of the plasma membrane. These subtractions resemble, in low buoyant density, structures of the plasma membrane termed caveolae. Immunofluorescence experiments revealed a punctate pattern of G protein α and β subunits, consistent with concentration of these proteins at distinct sites on the plasma membrane. Partial coincidence of localization of G protein α subunits with caveolin (a marker for caveolae) was observed by double immunofluorescence. Results of immunogold electron microscopy suggest that some G protein is associated with invaginated caveolae, but most of the protein resides in irregular structures of the plasma membrane that could not be identified morphologically. Because regulated adenylyl cyclase activity is present in low-density subtractions of plasma membrane from a cell type (S49 lymphoma) that does not express caveolin, this protein is not required for organization of the adenylyl cyclase system. The data suggest that hormone-sensitive adenylyl cyclase systems are localized in a specialized subdomain of the plasma membrane that may optimize the efficiency and fidelity of signal transduction.

3.20 Platelet-derived growth factor activates mitogen-activated protein kinase in isolated caveolae
Liu, P., Ying, Y-S., and Anderson. R.G.W.
Proc. Natl. Acad. Sci., USA, ***94****, 13666-13670 (1997)*

The ability of a peptide hormone to affect many different intracellular targets is thought to be possible because of the modular organization of signal transducing molecules in the cell. Evidence for the presence of signaling modules in metazoan cells, however, is incomplete. Herein we show, with morphology and cell fractionation, that all the components of a mitogen-activated protein kinase pathway are concentrated in caveolae of unstimulated human fibroblasts. Addition of platelet-derived growth factor to either the intact cell or caveolae isolated from these cells stimulates tyrosine phosphorylation and activates mitogen-activated protein kinases in caveolae. The molecular machinery for kinase activation, therefore, is preorganized at the cell surface of quiescent cells.

3.21 Aminopeptidase I is targeted to the vacuole by a nonclassical vesicular mechanism
Scott, S.V., Baba, M., Ohsumi, Y. and Klionsky, D.J.
J. Cell Biol., ***138(1)****, 37-44 (1997)*

The yeast vacuolar protein aminopeptidase I (API) is synthesized as a cytosolic precursor that is transported to the vacuole by a nonclassical targeting mechanism. Recent genetic studies indicate that the biosynthetic pathway that transports API uses many of the same molecular components as the degradative autophagy pathway. This overlap coupled with both in vitro and in vivo analysis of API import suggested that, like autophagy, API transport is vesicular. Subcellular fractionation experiments (**OptiPrep**) demonstrate that API precursor (prAPI) initially enters a nonvacuolar cytosolic compartment. In addition, subvacuolar vesicles containing prAPI were purified from a mutant strain defective in breakdown of autophagosomes, further indicating that prAPI enters the vacuole inside a vesicle. The purified subvacuolar vesicles do not appear to contain vacuolar marker proteins. Immunogold EM confirms that prAPI is localized in cytosolic and in subvacuolar vesicles in a mutant strain defective in autophagic body degradation. These data suggest that the cytosolic vesicles containing prAPI fuse with the vacuole to release a membrane-bounded intermediate compartment that is subsequently broken down, allowing API maturation.

3.22 Bisecting GlcNac structures act as negative sorting signals for cell surface Glycoproteins in forskolin-treated rat hepatoma cells
Sultan, A.S. et al
J. Biol. Chem., ***272(5)****, 2866-2872 (1997)*

The bisecting N-acetylglucosamine residue is formed by UDP-N-acetylglucosamine:β-D-mannoside-β-1,4-N-acetylglucosaminyltransferase III (GnT-III), a key branching enzyme for N-glycans. We found that forskolin, an adenylyl cyclase activator, markedly enhanced GnT-III at the transcriptional level in various hepatoma cells and hepatocytes, resulting in an increase of bisecting GlcNAc residues in various glycoproteins, as judged from the lectin binding to erythroagglutinating phytohemagglutinin (E-PHA). In whole cell lysates, the E-PRA binding was increased, and leukoagglutinating phytohemagglutinin (L-PHA) binding was decreased at 12 h after forskolin treatment, by time, both GnT-III activity and mRNA had reached the maximum levels. In contrast, the binding capacity as to E-PHA, determined by fluorescence-activated cell sorting on the cell surface, was decreased, suggesting that bisecting GlcNAc structures in certain glycoproteins changed the expression levels of glycoproteins and decreased their sorting on the cell surface. Fractionated organelles of M31 cells showed that the binding capacity as to E-PHA was mainly localized in Golgi membranes and lysosomes. This was also supported by a fluorescence microscopy. In order to determine whether or not the bisecting GlcNAc residue acts as a sorting signal for glycoproteins, N-oligosaccharide structures of lysosomal-associated membrane glycoprotein 1 and β-glucuronidase, γ-glutamyltranspeptidase, and secretory glycoproteins such as ceruloplasmin and α-fetoprotein were measured by E-PHA and L-PHA blotting after immunoprecipitation. The expression levels of lysosomal membrane glycoprotein 1 and γ-glutamyltranspeptidase on the cell surface were decreased at 12 h after forskolin treatment, indicating that the bisecting GlcNAc structure may act as a negative sorting signal for the cell surface glycoproteins and may alter the characteristics of hepatoma cells. This is the first report on glycoprotein sorting related to a specific structure of oligosaccharides, bisecting GlcNAc.

3.23 Rapid plasma membrane anchoring of newly synthesized $p59^{fyn}$. Selective requirement for NH_2-terminal myristoylation and palmitoylation at cysteine-3.
van't Hof, W. and Resh, M.D.
J. Cell Biol., ***136(5)****, 1023-1035 (1997)*

Abstract. The trafficking of Src family proteins after biosynthesis is poorly defined. Here we studied the role of dual fatty acylation with myristate and palmitate in biosynthetic transport of $p59^{fyn}$. Metabolic labeling of transfected COS or NIH 3T3 cells with [^{35}S]methionine followed by analysis of cytosolic and total membrane fractions showed that Fyn became membrane bound within 5 min after biosynthesis. Newly synthesized Src, however, accumulated in the membranes between 20-60 min. Northern blotting detected Fyn mRNA specifically in soluble polyribosomes and soluble Fyn protein was only detected shortly (1-2 min) after radiolabeling. Use of chimeric Fyn and Src constructs showed that rapid membrane targeting was mediated by the myristoylated NH_2-terminal sequence of Fyn and that a cysteine at position 3, but not 6, was essential. Examination of $G\alpha_0$-, $G\alpha_s$-, or GAP43-Fyn fusion constructs indicated that rapid membrane anchoring is exclusively conferred by the combination of N-myristoylation plus palmitoylation of cysteine-3. Density gradient analysis colocalized newly synthesized Fyn with plasma membranes. Interestingly, a 10-20-min lag phase was observed between plasma membrane binding and the acquisition of non-ionic detergent insolubility. We propose a model in which synthesis and myristoylation of Fyn occurs on soluble ribosomes, followed by rapid palmitoylation and plasma membrane anchoring, and a slower partitioning into detergent-insoluble membrane subdomains. These results serve to define a novel trafficking pathway for Src family proteins that are regulated by dual fatty acylation.

3.24 Molecular cloning and expression of a chloride ion channel of cell nuclei
Valenzuela, S.M. et al
*J. Biol. Chem., **272(19)**, 12575-12582 (1997)*

Ion channels are known to be present on the plasma membrane of virtually all cells and have been found on the membranes of various intracellular organelles. However, until recently they were believed not to occur at the nuclear membrane. In this study we describe the molecular cloning and characterization of a nuclear ion channel protein, designated nuclear chloride channel-27 (NCC27), from the human myelomonocytic cell line, U937. NCC27 is a novel chloride ion channel protein that was found to localize principally to the cell nucleus. Its only known homologue is a bovine chloride ion channel protein (p64) believed to localize to internal organelles. NCC27 therefore represents the first human member of a new class of organellar chloride ion channel proteins.

3.25 Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Gα subunits $G\alpha_q$ and $G\alpha_i$ in caveolae in DDT_1 MF-2 smooth muscle cells
de Weerd, W.F.C. and Leeb-Lundberg, L.M.F.
*J. Biol. Chem., **272(28)**, 17858-17866 (1997)*

In this report, we show that the vasoactive peptide agonist bradykinin (BK) when bound to B2 BK receptors on DDT_1MF-2 smooth muscle cells promotes the recruitment and sequestration of the occupied receptors and the receptor-coupled G-protein a subunits $G\alpha_q$ and $G\alpha_i$ in caveolae. Association of ligand receptor complexes and Gα subunits with caveolae was indicated by their co-enrichment on density gradients with caveolin, a marker protein for caveolae. Caveolin and Gα subunits were monitored by immunoblotting, whereas receptors were monitored as ligand receptor complexes formed by labeling receptors with the agonist BK or the antagonist NPC17731 prior to cell disruption and caveolae enrichment. These complexes were detected with radioligand and by immunoblotting with BK antibodies. A direct interaction of Gα subunits with caveolin was also indicated by their co-immunoprecipitation. Immunoelectron microscopy revealed that the enriched caveolin, Gα subunits, and BK receptor complexes were present in structures of 0.1-0.2 μm. At 4°C, BK and NPC17731 receptor complexes were detected in caveolae, and both complexes were sensitive to acid washing prior to cell disruption and caveolae enrichment. Elevation of the temperature to 37°C increased the amount of BK receptor complexes in caveolae with a maximal response at 10 min (continuous labeling) or 20 min (single-round labeling), and the complexes became acid-resistant. These conditions also increased the amount of $G\alpha_q$ and $G\alpha_i$. in caveolae with a maximal response at 5-10 min. In contrast, the NPC17731 receptor complexes remained acid sensitive and dissociated at this temperature, and antagonists did not increase the amount of Gα subunits in caveolae. These results show that some agonists that act through G-protein-coupled receptors promote the association of their receptors and receptor-coupled Gα subunits with caveolae.

3.26 Vacuoles induced by *Helicobacter pylori* toxin contain both late endosomal and lysosomal markers
Molinari, M.et al
*J. Biol. Chem., **272(40)**, 25339-25344 (1997)*

Intoxication of mammalian cells with the vacuolating toxin (VacA) released by *Helicobacter pylori* causes the formation of large acidic vacuoles containing the vacuolar ATPase proton pump and Rab7, a late endosome marker. Here, we describe a novel subcellular fractionation procedure, and we show that nanomolar concentrations of VacA induce a clear redistribution of lysosomal membrane glycoproteins among endocytic compartments. This redistribution is an early event in the process of cellular intoxication by VacA and precedes the formation of macroscopic vacuoles. The absence of the cation independent mannose 6-P receptor and the presence of Rab7 and of lysosomal membrane proteins in the newly formed compartment suggest that the vacuolating toxin induces the accumulation of a post-endosomal hybrid compartment presenting both late endosomal and lysosomal features.

3.27 Inhibition of endosome function in CHO cells bearing a temperature-sensitive defect in the coatomer (COPI) component ∈-COP

Daro, E., Sheff, D., Gomez, M., Kreis, T. and Mellman, I.
J. Cell Biol., ***139(7)***, *1747-1759 (1997)*

Abstract. Recent evidence has suggested that subunits of the coatomer protein (COPI) complexes are functionally associated with endosomes in mammalian cells. We now provide genetic evidence that COPI plays a role in endocytosis in intact cells. The 1D1F mutant CHO cell line bears a temperature-sensitive defect in the COPI subunit ∈-COP**.** In addition to exhibiting conditional defects in the secretary pathway, we find that the cells are also defective at mediating endosome-associated functions. As found for cells microinjected with anti-COPI antibodies, 1D1F cells at the restrictive temperature could not be infected by vesicular stomatitis (VSV) or Semliki Forest virus (SFV) that require delivery to acidic endosomes to penetrate into the cytosol. Although there was no temperature-sensitive defect in the internalization of receptor-bound transferrin (Tfn), Tfn recycling and accumulation of HRP were markedly inhibited at the restrictive temperature. Sorting of receptor-bound markers such as EGF to lysosomes was also reduced, although delivery of fluid-phase markers was only partially inhibited. In addition, lysosomes redistributed from their typical perinuclear location to the tips of the 1D1F cells. Mutant phenotypes began to emerge within 2 h of temperature shift, the time required for the loss of detectable ∈-COP**,** suggesting that the endocytic defects were not secondary to a block in the secretary pathway. Importantly, the mutant phenotypes were also corrected by transfection of wild-type ∈-COP cDNA demonstrating that they directly or indirectly reflected the ∈-COP defect. Taken together, the results suggest that ∈-COP acts early in the endocytic pathway, most likely inhibiting the normal sorting and recycling functions of early endosomes.

3.28 Identification of caveolin and caveolin-related proteins in the brain

Cameron, P.L., Ruffin, J.W., Bollag, R., Rasmussen, H. and Cameron, R.S.
J. Neurosci., ***17(24)***, *9520-9535 (1997)*

Caveolae are 50-100 nm, nonclathrin-coated, flask-shaped plasma membrane microdomains that have been identified in most mammalian cell types, except lymphocytes and neurons. To date, multiple functions have been ascribed to caveolae, including the compartmentalization of lipid and protein components that function in transmembrane signaling events, biosynthetic transport functions, endocytosis, potocytosis, and transcytosis. Caveolin, a 21-24 kDa integral membrane protein, is the principal structural component of caveolae. We have initiated studies to examine the relationship of detergent-insoluble complexes identified in astrocytes to the caveolin-caveolae compartment detected in cells of peripheral tissues. Immunolocalization studies performed in astrocytes reveal caveolin immunoreactivity in regions that correlate well to the distribution of caveolae and caveolin determined in other cell types, and electron microscopic studies reveal multiple clusters of flask-shaped invaginations aligned along the plasma membrane. Immunoblot analyses demonstrate that detergent insoluble complexes isolated from astrocytes are composed of caveolin-1 a, an identification verified by Northern blot analyses and by the cloning of a CDNA using reverse transcriptase-PCR amplification from total astrocyte RNA. Using a full-length caveolin-1 probe, Northern blot analyses suggest that the expression of caveolin-1 may be regulated during brain development. Immunoblot analyses of detergent-insoluble complexes isolated from cerebral cortex and cerebellum identify two immunoreactive polypeptides with apparent molecular weight and isoelectric points appropriate for caveolin. The identification of caveolae microdomains and caveolin-1 in astrocytes and brain, as well as the apparent regulation of caveolin-1 expression during brain development, identifies a cell compartment not detected previously in brain.

3.29 High-density-lipoprotein subfraction 3 interaction with glycosylphosphatidyl-inositol-anchored proteins

Nion, S. et al

*Biochem. J., **328**, 415-423 (1997)*

To elucidate further the binding of high-density-lipoprotein subfraction 3 (HDL_3) to cells, the involvement of glycosylphosphatidylinositol-anchored proteins (GPI-proteins) was studied. Treatment of cultured cells, such as fibroblasts or SK-MES-1 cells, with a phosphatidylinositol-specific phospholipase C(PI-PLC) significantly decreases specific HDL_3 binding. Moreover, PI-PLC treatment of cultured cells or cellular plasma membrane fractions results in releasing proteins. These proteins have a soluble form and can also bind HDL_3, as revealed by ligand blotting experiments with HDL_3. In order to obtain enriched GPI-proteins, we used a detergent-free purification method to prepare a caveolar membrane fraction. In the caveolar fraction, we obtained, by ligand blotting experiments, the enrichment of two HDL_3-binding proteins with molecular masses of 120 and 80 kDa. These proteins were also revealed in a plasma membrane preparation with two other proteins, with molecular masses of 150 and 104 kDa, and were sensitive to PI-PLC treatment. Electron microscopy also showed the binding of Au-labelled HDL_3 inside the vacuolar membrane invaginations. In SK-MES-1 cells, HDL_3 are internalized into a particular structure, resulting in the accumulation and concentration of such specific membrane domains. To sum up, a demonstration has been made of the implication of GPI-proteins as well as caveolae in the binding of HDL_3 to cells.

3.30 Reduction of protein disulfide bonds in an oxidizing environment

Majoul, I., Ferrari, D. and Soling, H-D.

*FEBS Lett., **401**, 104-108 (1997)*

Following retrograde transport to the endoplasmic reticulum (ER) the A-subunit of cholera toxin (CTX-A) is partially cleaved into CTX-A1 and CTX-A2 by reduction of a disulfide bridge [Majoul et al (1996) J. Cell Biol. 133, 777-789], although the redox state in the ER favors disulfide formation. We show here that the disulfide bridge of CTX-A is cleaved in vitro already at GSH/GSSG ratios between 1 and 3. Protein disulfide isomerase (PDI) exerts only a minor accelerating effect. Various mixed disulfide intermediates (CTX-A1-S-S-CTX-A1; PDI-S-S-A2; PDI-S-S-A1) appear during CTX-A reduction. These results indicate that in the ER protein disulfide formation and protein disulfide reduction can take place simultaneously.

3.31 Calbindin-D_{28k} in nerve cell nuclei

German, D.C., Ng, M.C., Liang, C.L., McMahon, A and Iacopino, A.M.

*Neurosci., **81(3)**, 735-743 (1997)*

Calbindin-D_{28k} is a member of the large EF-hand family of calcium-binding proteins that is believed to function, in part, as a cytosolic calcium buffer. Recent studies have demonstrated that cells containing Calbindin-D_{28k} are protected from degeneration caused by conditions that elevate intracellular calcium concentration. Since its initial discovery in 1966, Calbindin-D_{28k} has been localized in the cytoplasm of many neuronal populations, but its nuclear localization has been uncertain. Using light and electron microscopic immunochemistry, and nuclear fractionation methods, we demonstrate localization of Calbindin-D_{28k} not only in the cytoplasm, but also in the nucleus of rodent midbrain dopaminergic neurons and cerebellar Purkinje cells. The Calbindin-D_{28k} immunoreactive staining intensity in the nucleus was routinely equal or greater than that in the cytoplasm. Since calcium signals are propagated to the nucleus, where they can regulate gene expression, the existence of nuclear Calbindin-D_{28k} has important implications for cellular functions.

3.32 Membrane association of FtsY, the *E. coli* SRP receptor

DeLeeuw, et al

*FEBS Lett., **416**, 225-229 (1997)*

FtsY, the *Escherichia coli* homologue of the eukaryotic SRP receptor (SRα), is located both in the cytoplasm and in the inner membrane of *E. coli*. Similar to SRα, FtsY consists of the two major domains: a strongly acidic N-terminal domain (A) and a C-terminal GTP binding domain (NG) of which the crystal structure has recently been determined. The domains were expressed both in vivo and in vitro to examine their subcellular localization. The results suggest that both domains associated with the membrane but that the nature of the association differs.

3.33 Role for the target enzyme in deactivation of photoreceptor G protein in vivo
Tsang, S.H., et al**.**
*Science, **282(5386)**, 117-121(1998)*

Heterotrimeric guanosine 5'-triphosphate (GTP)-binding proteins (G proteins) are deactivated by hydrolysis of the GTP that they bind when activated by transmembrane receptors. Transducin, the G protein that relays visual excitation from rhodopsin to the cyclic guanosine 3', 5'-monophosphate phosphodiesterase (PDE) in retinal photoreceptors, must be deactivated for the light response to recover. A point mutation in the γ subunit of PDE impaired transducin-PDE interactions and slowed the recovery rate of the flash response in transgenic mouse rods. These results indicate that the normal deactivation of transducin in vivo requires the G protein to interact with its target enzyme.

3.34 Characterization of a cytosolic heat-shock protein-caveolin chaperone complex.
Uittenbogaard, A., Ying, Y.S. and Smart, E.J.
*J. Biol. Chem., **273(11)**, 6525-6532, (1998)*

Caveolin is a 22-kDa protein that appears to play a critical role in regulating the cholesterol concentration of caveolae. Even though caveolin is thought to be a membrane protein, several reports suggest that this peculiar protein can traffic independently of membrane vesicles. We now present evidence that a cytosolic pool of caveolin is part of a heat-shock protein-immunophilin chaperone complex consisting of caveolin, heat-shock protein 56, cyclophilin 40, cyclophilin A, and cholesterol. Treatment of NIH 3T3 cells with 1 μm cyclosporin A or 100 nm rapamycin disrupted the putative transport complex and prevented rapid (10-20 min) transport of cholesterol to caveolae. The lymphoid cell line, L1210-JF, does not express caveolin, does not form an immunophilin-caveolin complex, and does not transport newly synthesized cholesterol to caveolae. Transfection of caveolin cDNA into L1210-JF cells allowed the assembly of a transport complex identical to that found in NIH 3T3 cells. In addition, newly synthesized cholesterol in transfected cells was rapidly (10-20 min) and specifically transported to caveolae. These data strongly suggest that a caveolin-chaperone complex is a mechanism by which newly synthesized cholesterol is transported from the endoplasmic reticulum through the cytoplasm to caveolae.

3.35 Dissection of hepatic receptor-mediated endocytic pathways using self-generated gradients of iodixanol (OptiPrep).
Billington, D., Maltby, P.J. Jackson, A.P. and Graham, J.M.
*Anal. Biochem., **258**, 251-258 (1998).*

Iodixanol is a new, non-ionic, iodinated density gradient medium which has the advantage over other similar media in that it rapidly forms self-generated gradients in vertical or near-vertical rotors. Endocytosis of ^{99m}Tc-labelled neogalactosyl albumin (^{99m}Tc-NGA), a synthetic ligand for the asialoglycoprotein receptor, was studied by administering the ligand as a short pulse to perfused rat livers operating under single pass conditions. Intracellular processing was arrested at various times after the pulse and the resultant homogenate cleared of nuclei and heavy mitochondria by centrifugation at 3000g for 10 min. After adjusting to 12.5% (w/v) iodixanol, the 3000g supernatants were centrifuged at 350,000g for 60 min to form the gradients in which early, clathrin-containing vesicles, low-density endosomes and lysosomes were well-resolved. ^{99m}Tc-NGA bound to the sinusoidal membrane could be partially resolved from clathrin-containing vesicles by inclusion of 1 mM $CaCl_2$ in the homogenisation and gradient buffers. Two populations of early clathrin-containing vesicles could be resolved by rate-zonal centrifugation in pre-formed iodixanol gradients. Thus, iodixanol is an excellent density gradient medium for the rapid and efficient resolution of endosome compartments.

3.36 Subcellular distribution and turnover of presenilins in transfected cells.
Zhang, J. et al
*J. Biol. Chem., **273(20)**, 12436-12442 (1998)*

The mechanisms by which mutations in presenilin-1 (PS1) and presenilin-2 (PS2) result in the Alzheimer=s disease phenotype are unclear. Full-length PS1 and PS2 are each processed into stable proteolytic fragments after their biosynthesis in transfected cells. PS1 and PS2 have been localized by immunocytochemistry to the endoplasmic reticulum (ER) and Golgi compartments, but previous studies could not differentiate between the full-length presenilins and their fragments. Full-length PS1 and PS2 were principally distributed in ER fractions, whereas the N- and C-terminal fragments were localized predominantly to the Golgi fractions. In cells expressing the PS1 mutant lacking exon (ΔE9), we observed only full-length molecules that were present

in the ER and Golgi fractions. The turnover rate was considerably slower for the ΔE9 holoprotein, apparently due to decreased degradation within the ER. Our results suggest that full-length presenilin proteins are primarily ER resident molecules and undergo endoproteolysis within the ER. The fragments are subsequently transported to the Golgi compartment, where their turnover rate is much slower than that of the full-length presenilin in the ER.

3.37 Dietary fish oils modify the assembly of VLDL and expression of the LDL receptor in rabbit liver.
Wilkinson, J., Higgins, J.A., Fitzsimmons, C. and Bowyer, D.E.
*Arterioscler. Thromb. Vasc. Biol., **18**, 1490-1497 (1998)*

Supplementation of the diet of rabbits with fish oil or sunflower oil resulted in significant changes in the lipoproteins and lipids in serum. Compared with chow-fed rabbits, dietary fish oils decreased very low density lipoprotein (VLDL), increased low density lipoprotein (LDL), and shifted the peak of the LDL to denser fractions, whereas sunflower oil increased high density lipoprotein and shifted LDL to the lighter fractions. The amount of LDL receptors in fish oil-fed rabbit liver decreased by > 70% while there was only a small fall in these levels in sunflower oil-fed rabbit liver. The concentrations of apolipoprotein (apo) B in the subcellular organelles of the secretory compartment (rough and smooth endoplasmic reticula and Golgi fractions) were also changed by dietary lipids. In both sunflower oil- and fish oil-fed liver, apo B was increased in the lumen of the rough endoplasmic reticulum compared with fractions from chow-fed rabbit liver. The apo B in the trans-Golgi lumen from fish oil-fed livers was reduced and occurred in particles of $d \approx 1.21$ g/mL. In contrast, apo B in the trans-Golgi lumen from livers of sunflower oil-fed rabbits was increased and occurred in particles of $d < 1.21$ g/mL. These results suggests that feeding of fish oils causes an interruption in the intracellular transfer of apo B and hence assembly of VLDL. This leads to an enrichment of the rough endoplasmic reticulum membranes with cholesterol, thus down regulating the expression of the LDL receptor.

3.38 Phosphatidylinositol 4-phosphate synthesis in immunoisolated caveolae-like vesicles and low buoyant non-caveolar membranes.
Waugh, M.G., Lawson, D., Tan, S.K. and Hsuan, J.J.
*J. Biol. Chem., **273(27)**, 17115-17121 (1998)*

This study examined phosphatidylinositol 4-phosphate (PtdIns4P) synthesis in caveolae that have been suggested to be discrete signaling microdomains of the plasma membrane and are enriched in the marker protein caveolin. Caveolin-rich light membranes (CLMs) were isolated from A431 cells by detergent-free, discontinuous density-gradient centrifugation method. The CLM fraction was separated from the bulk of the cellular protein and was greatly enriched in PtdIns, PtdIns4P, and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and an adenosine-sensitive type II PtdIns 4-kinase activity. Preparation of CLMs by an **OptiPrep**-based cell fractionation procedure confirmed the co-localization of PtdIns 4-kinase and caveolin. Electron microscopy confirmed that an anti-caveolin antiserum immunopurified vesicles from CLMs that were within the size range described for caveolae in other systems. Co-immunoprecipitated PtdIns 4-kinase activity could utilize endogenous PtdIns, present within the caveolae and CLMs. However, less than 1% of the total cellular PtdIns and PtdIns 4-kinase activity was present in caveolae-like vesicles, indicating that non-caveolar light membranes rafts are the main sites for cellular PtdIns4P production.

3.39 Rapid enzyme-free preparation of starch-free nuclei from plants facilitates studies of chromatin structure.
Ford, T.C., Baldwin, J.P. and Lambert, S.J.
Plant proteins in abiotic stress responses, Plant Protein Club, 1998 Annual Symposium, University of York, p24 (1998)

Several studies of the chromatin structure of normal or stressed plant cells require rapid purification of the cell nuclei under conditions which do not alter the structure of the active or inactive genes under investigation. We have developed a very simple, rapid and enzyme-free method for preparing wheatgerm nuclei by centrifugation on an **iodixanol** step between densities 1.168 and 1.234 g/ml with a minimal contribution of the iodixanol to solution colligative properties (osmotic pressure). Histone octamers, which are key molecular complexes in the regulation of transcription and which are acetylated in specific lysines in active genes have been purified rapidly from the pure nuclei.

3.40 Insulin-induced protein tyrosine phosphorylation cascade and signalling molecules are localized in a caveolin-enriched cell membrane domain.
Smith, R.M., Harada, S., Smith, J.A., Zhang, S. and Jarett, L.
*Cell. Signalling, **10(5)**, 355-362 (1998)*

The cellular localisation of time- and temperature-dependent ^{125}I-insulin binding, insulin-sensitive signalling proteins and the insulin-induced protein tyrosine phosphorylation cascade were assessed in subcellular fractions isolated on **Iodixanol** gradients from control and insulin-treated H35 hepatoma cells. Western blot analysis demonstrated that the concentrations of IRS-1, Shc, GRB-2, SOS, Syp, PI 3-kinase, MAP kinase and $G_{1\alpha}$ were at least 10-fold higher in cell surface-derived, caveolin-enriched fraction than in a cell surface-derived, caveolin-poor fraction (i.e., the plasma membranes). Insulin treatment caused a 15-fold increase in tyrosine phosphorylation of IRS-1 in the caveolin-enriched fraction in 5 min at 37°C compared with a 3-fold increase in plasma membranes and a 6-fold increase in the cytosol and endosomes. Insulin also increased tyrosine phosphorylation of both a 72-kDa protein and the 46-kDa Shc isoform only in the caveolin-enriched fraction. Insulin treatment did not change the concentrations of insulin receptors or Shc but increased IRS-1 in the caveolin-enriched fraction, possibly recruited from the cytosolic pool. Insulin also increased the concentrations of insulin receptors, IRS-1 and Shc in endosomes, suggesting insulin-induced internalization of the insulin receptors and proteins activated with them. Electron microscopic analysis, with the use of a combination of colloidal gold-labelled insulin to label the insulin receptor and immunolabelling to detect caveolin or IRS-1, demonstrated the co-localisation of insulin receptors in caveolin- and IRS-1 containing vesicular structures. Differences in the insulin-induced protein tyrosine phosphorylation and concentrations of these proximal signalling proteins in the caveolin-enriched fraction, plasma membranes, and cytosol suggest that insulin receptors in the caveolae play a major role in the initiating insulin's signal transduction processes.

3.41 Purification and characterization of autophagosomes from rat hepatocytes.
Strømhaug, P.E., Berg, T.O.,
and Seglen, P.O.
*Biochem. J., **335**, 217-224 (1998)*

To investigate the properties and intracellular origin of autophagosomes, a procedure for the purification and isolation of these organelles from rat liver has been developed. Isolated hepatocytes were incubated with vinblastine to induce autophagosome accumulation; the cells were then homogenized and treated with the cathepsin C substrate glycyl-1-phenylalanine 2-naphthylamide to cause osmotic disruption of the lysosomes. Nuclei were removed by differential centrifugation, and the postnuclear supernatant was fractionated on a discontinuous Nycodenz density gradient. The autophagosomes, recognized by their content of autophagocytosed lactate dehydrogenase (LDH), could be recovered in an intermediated-density fraction, free from cytosol and mitochondria. Finally, the autophagosomes were separated from the endoplasmic reticulum and other membraneous elements by centrifugation in a Percoll colloidal density gradient, followed by flotation in **iodixanol** to remove the Percoll particles. The final autophagosome preparation represented a 24-fold purification of autophagocytosed LDH relative to intact cells, with a 12% recovery. The purified autophagosomes contained sequestered cytoplasm with a normal ultrastructure, including mitochondria, peroxisomes and endoplasmic reticulum in the same proportions as in intact cells. However, immunoblotting indicated a relative absence of cytoskeletal elements (tubulin, actin and cytokeratin), which may evade autophagic sequestration. The autophagosomes showed no enrichment in protein markers typical of lysosomes (acid phosphatase, cathepsin B, lysosomal glycoprotein of 120 kDa), endosomes (early-endosome-associated proteins 1, cation-independent mannose 6-phosphate-regulated protein of 78 kDa, protein disulphide isomerase), suggesting that the sequestering membranes are not derived directly from any of these organelles, but rather represent unique organelles (phagophores).

3.42 Presenilin 1 regulates the processing of β-amyloid precursor protein C-terminal fragments and the generation of amyloid β-protein in endoplasmic reticulum and Golgi.
Xia, W. et al
*Biochemistry, **37(47)**, 16465-71 (1998)*

Progressive cerebral deposition of the amyloid β-protein (Aβ) is believed to play a pivotal role in the pathogenesis of Alzheimer's disease (AD). The highly amyloidogenic 42-residue form of Aβ ($A\beta_{42}$) is the first species to be deposited in both sporadic and familial AD. Mutations in two familial AD-linked genes, presenilins 1 (PS1) and 2 (PS2), selectively increase the production of $A\beta_{42}$ in cultured cells and the brains of transgenic mice, and gene deletion of PS1 shows that it is required for normal gamma-secretase cleavage of the beta-amyloid precursor protein (APP) to generate Aβ. To establish the subcellular localization of the PS1

regulation of APP processing to Aβ, fibroblasts from PS1 wild-type (wt) or knockout (KO) embryos as well as Chinese hamster ovary (CHO) cells stably transfected with wt or mutant PS1 were subjected to subcellular fractionation on discontinuous **Iodixanol** gradients. APP C-terminal fragments (CTF) were markedly increased in both endoplasmic reticulum- (ER-) and Golgi-rich fractions of fibroblasts from KO mice; moreover, similar increases were documented directly in KO brain tissue. No change in the subcellular distribution of full-length APP was detectable in fibroblasts lacking PS1. In CHO cells, a small portion of APP, principally the N-glycosylated isoform, formed complexes with PS1 in both ER- and Golgi-rich fractions, as detected by coimmunoprecipitation. When the same fractions were analyzed by enzyme-linked immunosorbent assays for $A\beta_{total}$ and $A\beta_{42}$, $A\beta_{42}$ was the major Aβ species in the ER fraction ($A\beta_{42}$:$A\beta_{total}$ ratio 0.5-1.0), whereas absolute levels of both $A\beta_{42}$ and $A\beta_{40}$ were higher in the Golgi fraction and the $A\beta_{42}$:$A\beta_{total}$ ratio was 0.05-0.16 there. Mutant PS1 significantly increased $A\beta_{42}$ levels in the Golgi fraction. Our results indicate PS1 and APP can interact in the ER and Golgi, where PS1 is required for proper γ-secretase processing of APP CTFs, and that PS1 mutations augment $A\beta_{42}$ levels principally in Golgi-like vesicles.

3.43 Role of plasmalemmal caveolae in signal transduction

Shaul, P.W. and Anderson, R.G.W.
*Am. J. Physiol., **275**, 843-851 (1998)*

Caveolae are specialized plasmalemmal microdomains originally studied in numerous cell types for their involvement in the transcytosis of macromolecules. They are enriched in glycosphingolipids, cholesterol, sphingomyelin, and lipid-anchored membrane proteins, and they are characterized by a light buoyant density and resistance to solubilization by Triton X-100 at 4°C. Once the identification of the marker protein caveolin made it possible to purify this specialized membrane domain, it was discovered that caveolae also contain a variety of signal transduction molecules. This includes C protein-coupled receptors, G proteins and adenylyl cyclase, molecules involved in the regulation of intracellular calcium homeostasis, and their effectors including the endothelial isoform of nitric oxide synthase, multiple components of the tyrosine kinase-mitogen-activated protein kinase pathway, and numerous lipid signaling molecules. More recent work has indicated that caveolae further serve to compartmentalize, modulate, and integrate signaling events at the cell surface. This specialized plasmalemmal domain warrants direct consideration in future investigations of both normal and pathological signal transduction in pulmonary cell types.

3.44 Targeting of protein Kinase Cα to caveolae

Mineo, C., Ying, Y-S, Chapline, C., Jaken, S. and Anderson, R.G.W.
*J. Cell Biol., **141(3)**, 601-610 (1998)*

Previously, we showed caveolae contain a population of protein kinase Cα (PKCα) that appears to regulate membrane invagination. We now report that multiple PKC isoenzymes are enriched in caveolae of unstimulated fibroblasts. To understand the mechanism of PKC targeting, we prepared caveolae lacking PKCα and measured the interaction of recombinant PKCα with these membranes. PKCα bound with high affinity and specificity to caveolae membranes. Binding was calcium dependent, did not require addition of factors that activate the enzyme, and involved the regulatory domain of the molecule. A68-kD PKCα-binding protein identified as sdr (serum deprivation response) was isolated by interaction cloning and localized to caveolae. Antibodies against sdr inhibited PKCα binding. A 100-aminoacid sequence from the middle of sdr competitively blocked PKCα binding while flanking sequences were inactive. Caveolae appear to be a membrane site where PKC enzymes are organized to carry out essential regulatory functions as well as to modulate signal transduction at the cell surface.

3.45 SR-BII, an isoform of the scavenger receptor BI containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells

Webb, N.R. et al
*J. Biol. Chem., **273(24)**, 15241-15248 (1998)*

The scavenger receptor class B, type I (SR-BI), binds high density lipoprotein (HDL) and mediates selective uptake of cholesteryl ester from HDL and HDL-dependent cholesterol efflux from cells. We recently identified a new MRNA variant that differs from the previously characterized form in that the encoded C-terminal cytoplasmic domain is almost completely different. In the present study, we demonstrate that the mRNAs for mouse SR-El and SR-BII (previously termed SR-BI.2) are the

alternatively spliced products of a single gene. The translation products predicted from human, bovine, mouse, hamster, and rat cDNAs exhibit a high degree of sequence similarity within the SR-BII C-terminal domain (62-67% identity when compared with the human sequence), suggesting that this variant is biologically important. SR-BII protein represents approximately 12% of the total immunodetectable SR-BI/II protein in mouse liver. Subcellular fractionation of transfected Chinese hamster ovary cells showed that SR-BII, like SR-BI, is enriched in caveolae, indicating that the altered cytoplasmic tail does not affect targeting of the receptor. SR-BII mediated both selective cellular uptake of cholesteryl ether from HDL as well as HDL-dependent cholesterol efflux from cells, although with approximately 4-fold lower efficiency than SR-BI. *In vivo* studies using adenoviral vectors showed that SR-BII was relatively less efficient than SR-BI in reducing plasma HDL cholesterol. These studies show that SR-BII, an HDL receptor isoform containing a distinctly different cytoplasmic tail, mediates selective lipid transfer between HDL and cells, but with a lower efficiency than the previously characterized variant.

3.46 Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase (ERK)
Furuchi, T. and Anderson, R.G.W.
J. Biol. Chem., ***273(33)****, 21009-21104 (1998)*

Previously we showed that activation of Erk in quiescent cells occurs in the caveolae fraction isolated from fibroblasts. Since the structure and function of caveolae is sensitive to the amount of cholesterol in the membrane, it might be that a direct link exists between the concentration of membrane cholesterol and mitogen activated protein (MAP) kinase activation. We acutely lowered the cholesterol level of the caveolae fraction by incubating Rat-1 cells in the presence of either cyclodextrin or progesterone. Cholesterol-depleted caveolae had a reduced amount of several key protein components of the MAP kinase complex, including Ras, Grb2, Erk2, and Src. Incubation of these cells in the presence of epidermal growth factor (EGF) caused a rapid loss of EGF receptor from the caveolae fraction, but the usual recruitment of c-Raf was markedly inhibited. Despite the reduced amount of c-Raf and Erk2 in the cholesterol depleted caveolae fraction, EGF caused a hyperactivation of the remaining caveolae Erk isoenzymes. This was followed by an increase in the amount of active Erk in the cytoplasm. The increased amount of activated Erk produced under these conditions was linked to a 2-fold higher level of EGF-stimulated DNA synthesis. Even cholesterol depletion by itself stimulated Erk activation and DNA synthesis. These results suggest that the MAP kinase pathway can connect the cholesterol level of caveolae membrane to the control of cell division.

3.47 Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells
Grindstaff, K.K., Yeaman, C., Anandasabapathy, N., Hsu, S.C., Rodriguez-Boulan,E., Scheller R.H. and Nelson, W.J.
Cell, ***93****, 731-740 (1998)*

In budding yeast, the Sec6/8p complex is essential for generating cell polarity by specifying vesicle delivery to the bud tip. We show that Sec6/8 homologs are components of a cytosolic, ~17S complex in nonpolarized MDCK epithelial cells. Upon initiation of calcium-dependent cell-cell adhesion, ~70% of Sec6/8 is rapidly ($t_{1/2} \approx$ 3-6 hr) recruited to sites of cell-cell contact. In streptolyslin-O-permeabilized MDCK cells, Sec6/8 antibodies inhibit delivery of LDL receptor to the basal-lateral membrane, but not $p75^{NTR}$ to the apical membrane. These results indicate that lateral membrane recruitment of the Sec6/8 complex is a consequence of cell-cell adhesion and is essential for the biogenesis of epithelial cell surface polarity.

3.48 ARNO is a guanine nucleotide exchange factor for ADP-ribosylation factor 6
Frank, S., Upender, S., Hansen, S.H. and Casanova, J.E.
J. Biol. Chem., ***273(1)****, 23-27(1998)*

ADP-ribosylation factors (ARFS) constitute a family of small monomeric GTPases. ARFs 1 and 3 function in the recruitment of coat proteins to membranes of the Golgi apparatus, whereas ARF6 is localized to the plasma membrane, where it appears to modulate both the assembly of the actin cytoskeleton and endocytosis. Like other GTPases, ARF activation is facilitated by specific guanine nucleotide exchange factors (GEFs). ARNO (ARF nucleotide-binding site opener) is a member of a growing family of ARF-GEFs that share a common, tripartite structure consisting of an N-terminal coiled-coil domain, a central domain with homology to the yeast protein Sec7p, and a C-terminal pleckstrin homology domain. Recently, ARNO and its close homologue cytohesin-1 were found to catalyze *in vitro*

nucleotide exchange on ARF1 and ARF3, respectively, raising the possibility that these GEFs function in the Golgi. However, the actual function of these proteins may be determined in part by their ability to interact with specific ARFs and in part by their subcellular localization. We report here that *in vitro* ARNO can stimulate nucleotide exchange on both ARF1 and ARF6. Furthermore, based on subcellular fractionation and immunolocalization experiments, we find that ARNO is localized to the plasma membrane in mammalian cells rather than the Golgi. It is therefore likely that ARNO functions in plasma membrane events by modulating the activity of ARF6 *in vivo*. These findings are consistent with the previous observation that cytohesin-1 regulates the adhesiveness of $\alpha L\beta_2$ integrins at the plasma membrane of lymphocytes.

3.49 Retrograde transport of Golgi-localized proteins to the ER
Cole, N.B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwarz, J.
J. Cell Biol., ***140(1)****, 1-15 (1998)*

Abstract. The ER is uniquely enriched in chaperones and folding enzymes that facilitate folding and unfolding reactions and ensure that only correctly folded and assembled proteins leave this compartment. Here we address the extent to which proteins that leave the ER and localize to distal sites in the secretary pathway are able to return to the ER folding environment during their lifetime. Retrieval of proteins back to the ER was studied using an assay based on the capacity of the ER to retain misfolded proteins. The lumenal domain of the temperature-sensitive viral glycoprotein VSVGtsO45 was fused to Golgi or plasma membrane targeting domains. At the nonpermissive temperature, newly synthesized fusion proteins misfolded and were retained in the ER, indicating the VSVGtsO45 ectodomain was sufficient for their retention within the ER. At the permissive temperature, the fusion proteins were correctly delivered to the Golgi complex or plasma membrane, indicating the lumenal epitope of VSVGtsO45 also did not interfere with proper targeting of these molecules. Strikingly, Golgi-localized fusion proteins, but not VSVGtsO45 itself, were found to redistribute back to the ER upon a shift to the nonpermissive temperature, where they misfolded and were retained. This occurred over a time period of 15 min-2 h depending on the chimera, and did not require new protein synthesis. Significantly, recycling did not appear to be induced by misfolding of the chimeras within the Golgi complex. This suggested these proteins normally cycle between the Golgi and ER, and while passing through the ER at 40°C become misfolded and retained. The attachment of the thermosensitive VSVGtsO45 lumenal domain to proteins promises to be a useful tool for studying the molecular mechanisms and specificity of retrograde traffic to the ER.

3.50 Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport
Love, H.D., Lin, C.C., Short, C.S. and Ostermann, J.
J. Cell Biol., ***140(3)****, 541-551 (1998)*

Secretory proteins enter the Golgi apparatus when transport vesicles fuse with the *cis* -side and exit in transport vesicles budding from the *trans*-side. Resident Golgi enzymes that have been transported in the *cis-to-trans* direction with the secretary flow must be recycled constantly by retrograde transport in the opposite direction. In this study, we describe the functional characterization of Golgi-derived transport vesicles that were isolated from tissue culture cells. We found that under the steady-state conditions of a living cell, a fraction of resident Golgi enzymes was found in vesicles that could be separated from cistemal membranes. These vesicles appeared to be depleted of secretary cargo. They were capable of binding to and fusion with isolated Golgi membranes, and after fusion their enzymatic contents most efficiently processed cargo that had just entered the Golgi apparatus. Those results indicate a possible role for these structures in recycling of Golgi enzymes in the Golgi stack.

3.51 Caveolin-1 and –2 in the exocytic pathway of MDCK cells
Scheiffele, P. et al
J. Cell Biol., ***140(4)****, 795-806 (1998)*

Abstract. We have studied the biosynthesis and transport of the endogenous caveolins in MDCK cells. We show that in addition to homooligomers of caveolin-1, heterooligomeric complexes of caveolin-1 and -2 are formed in the ER. The oligomers become larger, increasingly detergent insoluble, and phosphorylated on caveolin-2 during transport to the cell surface. In the TGN caveolin-l/-2 heterooligomers are sorted into basolateral vesicles, whereas larger caveolin-I homooligomers are targeted to the apical side. Caveolin-1 is present on both the apical and basolateral plasma membrane, whereas caveolin-2 is enriched on the basolateral surface where caveolae are present. This suggests that caveolin-1 and -2 heterooligomers are involved in caveolar biogenesis in the basolateral plasma membrane. Anti-

caveolin-1 antibodies inhibit the apical delivery of influenza virus hemagglutinin without affecting basolateral transport of vesicular stomatitis virus G protein. Thus, we suggest that caveolin-1 homooligomers play a role in apical transport.

3.52 Caveolae, plasma membrane microdomains for α-secretase-mediated processing of the amyloid precursor protein

Ikezu, T et al

*J. Biol. Chem., **273(17)**, 10485-10495 (1998)*

Caveolae are plasma membrane invaginations where key signaling elements are concentrated. In this report, both biochemical and histochemical analyses demonstrate that the amyloid precursor protein (APP), a source of Aβ amyloid peptide, is enriched within caveolae. Caveolin-1, a principal component of caveolae, is physically associated with APP, and the cytoplasmic domain of APP directly participates in this binding. The characteristic C-terminal fragment that results from APP processing by α-secretase, an as yet unidentified enzyme that cleaves APP within the Aβ amyloid sequence, was also localized within these caveolae-enriched fractions. Further analysis by cell surface biotinylation revealed that this cleavage event occurs at the cell surface. Importantly, α-secretase processing was significantly promoted by recombinant overexpression of caveolin in intact cells, resulting in increased secretion of the soluble extracellular domain of APP. Conversely, caveolin depletion using antisense oligonucleotides prevented this cleavage event. Our current results indicate that caveolae and caveolins may play a pivotal role in the α-secretase-mediated proteolysis of APP in *vivo.*

3.53 Lipid domain structure of the plasma membrane revealed by patching of membrane components

Harder, T., Scheiffele, P., Verkade, P. and Simons K.

*J. Cell Biol., **141(4)**, 929-942 (1998)*

Abstract. Lateral assemblies of glycolipids and cholesterol, "rafts," have been implicated to play a role in cellular processes like membrane sorting, signal transduction, and cell adhesion. We studied the structure of raft domains in the plasma membrane of non-polarized cells. Overexpressed plasma membrane markers were evenly distributed in the plasma membrane. We compared the patching behavior of pairs of raft markers (defined by insolubility in Triton X-100) with pairs of raft/non-raft markers. For this purpose we cross-linked glycosyl-phosphatidylinositol (GPI)-anchored proteins placental alkaline phosphatase (PLAP), Thy-1, influenza virus hemagglutinin (HA), and the raft lipid ganglioside GM1 using antibodies and/or cholera toxin. The patches of these raft markers overlapped extensively in BHK cells as well as in Jurkat T-lymphoma cells. Importantly, patches of GPI-anchored PLAP accumulated src-like protein tyrosine kinase fyn, which is thought to be anchored in the cytoplasmic leaflet of raft domains. In contrast patched raft components and patches of transferrin receptor as a non-raft marker were sharply separated. Taken together, our data strongly suggest that coalescence of cross-linked raft elements is mediated by their common lipid environments, whereas separation of raft and non-raft patches is caused by the immiscibility of different lipid phases. This view is supported by the finding that cholesterol depletion abrogated segregation. Our results are consistent with the view that raft domains in the plasma membrane of non-polarized cells are normally small and highly dispersed but that raft size can be modulated by oligomerization of raft components.

3.54 Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons

Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C.G. and Simons, K.

*Proc. Natl. Acad. Sci. USA, **95**, 6460-6464 (1998)*

The amyloid precursor protein (APP) plays a crucial role in the pathogenesis of Alzheimer's disease. During intracellular transport APP undergoes a series of proteolytic cleavages that lead to the release either of an amyloidogenic fragment called β-amyloid (Aβ) or of a nonamyloidogenic secreted form consisting of the ectodomain of APP (APP_{sec}). It is Aβ that accumulates in the brain lesions that are thought to cause the disease. By reducing the cellular cholesterol level of living hippocampal neurons by 70% with lovastatin and methyl-β-cyclodextrin, we show that the formation of Aβ is completely inhibited while the generation of APP_{sec} is unperturbed. This inhibition of Aβ formation is accompanied by increased solubility in the detergent Triton X-100 and is fully reversible by the readdition of cholesterol to previously depleted cells. Our results show that cholesterol is required for Aβ formation to occur and imply a link between cholesterol, Aβ, and Alzheimer's disease.

3.55 CD14-dependent endotoxin internalization via a macropinocytic pathway

Poussin, C., Foti, M., Carpentier, J.L. and Pugin, J.
J. Biol. Chem., ***273(32)****, 20285-20291 (1998)*

Gram-negative bacterial endotoxin (a lipopolysaccharide (LPS)) specifically binds to CD14, a glycosylphosphatidyl inositol (GPI)-anchored surface myeloid glycoprotein. This interaction leads to cell activation, but it also promotes LPS internalization and detoxification. In this work, we investigated the route of LPS and CD14 internalization and the relevance of CD14 GPI anchor in the endocytic pathway. In promonocytic THP-1 ells transfected with a GPI or a chimeric integral form of CD14, we showed by differential buoyancy 'm sucrose density gradients that these two forms of CD14 were sorted to different plasma membrane subdomains. However, both forms of CD14 associated preferentially with the same surface microfilament-enriched microvilli or ruffles. Electron microscopic studies indicated that CD14 internalized via macropinocytosis, a process resembling that of phagocytosis, different from "classical" receptor-mediated endocytic pathways, such as clathrin-coated pits or caveolae. With cell warming, the CD14-enriched ruffles fused and formed large vesicles. Later, these vacuoles made stacks and condensed into phago-lysosomes. CD14 was specifically associated with all of these structures. Radiolabeled LPS internalization paralleled CD14 internalization. Confocal microscopic studies confirmed the co-localization of LPS and CD14 both at the cell surface and in endosomal compartments. The microfilament-disrupting, macropinocytosis blocking agent cytochalasin D inhibited LPS and CD14 internalization but did not prevent LPS-dependent activation, indicating that these two processes are dissociated.

3.56 Isolation and characterization of rat liver amphisomes

Berg, T.O., Fengsrud, M., Stromhaug, P.E., Berg, T. and Seglen, P.O.
J. Biol. Chem., ***273(34)****, 21883-21892 (1998)*

Amphisomes, the autophagic vacuoles (AVs) formed upon fusion between autophagosomes and endosomes, have so far only been characterized in indirect, functional terms. To enable a physical distinction between autophagosomes and amphisomes, the latter were selectively density-shifted in sucrose gradients following fusion with AOM-gold-loaded endosomes (endosomes made dense by asialoorosomucoid-conjugated gold particles, endocytosed by isolated rat hepatocytes prior to subcellular fractionation). Whereas amphisomes, by this criterion, accounted for only a minor fraction of the AVs in control hepatocytes, treatment of the cells with leupeptin (an inhibitor of lysosomal protein degradation) caused an accumulation of amphisomes to about one-half of the " population. A quantitative electron microscopic study confirmed that leupeptin induced a severalfold increase in the number of hepatocytic amphisomes (recognized by their gold particle contents; otherwise, their ultrastructure was quite similar to antophagosomes). Leupeptin caused, furthermore, a selective retention of endocytosed AOM-gold in the amphisomes at the expense of the lysosomes, consistent with an inhibition of amphisome-lysosome fusion. The electron micrographs suggested that autophagosomes could undergo multiple independent fusions, with multivesicular (late) endosomes to form amphisomes and with small lysosomes to form large autolysosomes. A biochemical comparison between autophagosomes and amphisomes, purified by a novel procedure, showed that the amphisomes were enriched in early endosome markers (the asialoglycoprotein receptor and the early endosome-associated protein 1) as well as in a late endosome marker (the cation-independent mannose 6-phosphate receptor). Amphisomes would thus seem to be capable of receiving inputs both from early and late endosomes.

3.57 Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast *Saccharomyces cerevisiae*

Kametaka, S., Okano, T., Ohsumi, M. and Ohsumi, Y.
J. Biol. Chem., ***273(35)****, 22284-22291 (1998)*

Mutation in the *Saccharomyces cerevisiae APG14* gene causes a defect in autophagy. Cloning and structural analysis of the APG14 gene revealed that APG14 encodes a novel hydrophilic protein with a predicted molecular mass of 40.5 kDa, and that Apgl4p has a coiled-coil motif at its N terminus region. We found that overproduction of Apgl4p partially reversed the defect in autophagy induced by the *apg6-1* mutation. The *apg6-1* mutant was found to be defective not only in autophagy but also in sorting of carboxypeptidase Y (CPY), a vacuolar-soluble hydrolase, to the vacuole. However, overexpression of *APG14* did not alter the CPY sorting defect of the *apg6-l* mutant, nor did the *apgl4* null mutation affect the CPY sorting pathway. Structural analysis of *APG6* revealed that *APG6* is identical to *VPS30*, which is involved in a retrieval step of the CPY receptor, Vpsl0p, to the late Golgi from the endosome (Seaman, M. N. J., Marcusson, E. G., Cereghino, J. L., and Emr, S. D. (1997) *J. Cell Biol* 137, 79-92). Subcellular

fractionation indicated that Apgl4p and Apg6p peripherally associated with a membrane structure(s). Apgl4p was co-immunoprecipitated with Apg6p, suggesting that they form a stable protein complex. These results imply that Apg6/Vps30p has two distinct functions in the autophagic process and the vacuolar protein sorting pathway. Apgl4p may be a component specifically required for the function of Apg6/Vps30p through the autophagic pathway.

3.58 Annexin XIIIb associates with lipid microdomains to function in apical delivery
Lafont, F., Lecat, S., Verkade, P. and Simons K.
J. Cell Biol., ***142(6)***, *1413-1427 (1998)*

A member of the annexin XIII sub-family, annexin XIIIb, has been implicated in the apical exocytosis of epithelial kidney cells. Annexins are phospholipid-binding proteins that have been suggested to be involved in membrane trafficking events although their actual physiological function remains open. Unlike the other annexins, annexin XIIIs are myristoylated. Here, we show by immunoelectron microscopy that annexin XIIIb is localized to the *trans*-Golgi network (TGN), vesicular carriers and the apical cell surface. Polarized apical sorting involves clustering of apical proteins into dynamic sphingolipid-cholesterol rafts. We now provide evidence for the raft association of annexin XIIIb. Using in vitro assays and either myristoylated or unmyristoylated recombinant annexin XIIIb, we demonstrate that annexin XIIIb in its native myristoylated form stimulates specifically apical transport whereas the unmyristoylated form inhibits this route. Moreover, we show that formation of apical carriers from the TGN is inhibited by an anti-annexin XIIIb antibody whereas it is stimulated by myristoylated recombinant annexin XIIIb. These results suggest that annexin XIIIb directly participates in apical delivery.

3.59 The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon
Valent, Q.A. et al
The EMBO J., ***17(9)***, *2504-2512 (1998)*

Two distinct protein targeting pathways can direct proteins to the *Escherichia coli* inner membrane. The Sec pathway involves the cytosolic chaperone SecB that binds to the mature region of pre-proteins. SecB targets the pre-protein to SecA that mediates preprotein translocation through the SecYEG translocon. The SRP pathway is probably used primarily for the targeting and assembly of inner membrane proteins. It involves the signal recognition particle (SRP) that interacts with the hydrophobic targeting signal of nascent proteins. By using a protein cross-linking approach, we demonstrate here that the SRP pathway delivers nascent inner membrane proteins at the membrane. The SRP receptor FtsY, GTP and inner membranes are required for release of the nascent proteins from the SRP. Upon release of the SRP at the membrane, the targeted nascent proteins insert into a translocon that contains at least SecA, SecY and SecG. Hence, as appears to be the case for several other translocation systems, multiple targeting mechanisms deliver a variety of precursor proteins to a common membrane translocation complex of the *E.coli* inner membrane.

3.60 Functions of lipid rafts in biological membranes
Brown, D.A. and London, E.
Annu. Rev. Cell Dev. Biol., ***14***, *111-136 (1998)*

Recent studies showing that detergent-resistant membrane fragments can be isolated from cells suggest that biological membranes are not always in a liquid-crystalline phase. Instead, sphingolipid and cholesterol-rich membranes such as plasma membranes appear to exist, at least partially, in the liquid-ordered phase or a phase with similar properties. Sphingolipid and cholesterol-rich domains may exist as phase-separated “rafts” in the membrane. We discuss the relationship between detergent-resistant membranes, rafts, caveolae, and low-density plasma membrane fragments. We also discuss possible functions of lipid rafts in membranes. Signal transduction through the high-affinity receptor for IgE on basophils, and possibly through related receptors on other hematopoietic cells, appears to be enhanced by association with rafts. Raft association may also aid in signaling through proteins anchored by glycosylphosphatidylinositol, particularly in hematopoietic cells and neurons. Rafts may also function in sorting and trafficking through the secretary and endocytic pathways.

3.61 The calcium-sensing receptor is localized in caveolin-rich plasma membrane domains of bovine parathyroid cells

Kifor, O., Diaz, R., Butters, R., Kifor, I. And Brown, E.M.

J. Biol. Chem., ***273(34)****, 21708-21713 (1998)*

Parathyroid cells have an intracellular machinery for parathyroid hormone (PTH) secretion that is inversely regulated by the extracellular calcium concentration (Ca^{2+}_o). The recently characterized Ca^{2+}_o sensing receptor (CaR) is a G protein-coupled, seven-transmembrane receptor mediating the inhibitory effects of high Ca^{2+}_o on PTH secretion. The CaR's precise cell surface localization and the signal transduction pathway(s) mediating its inhibitory effects on PTH secretion have not been characterized fully. Here, we demonstrate that the CaR resides within caveolin-rich membrane domains in bovine parathyroid cells. Chief cells within bovine parathyroid glands exhibit a similar pattern of staining for caveolin-1 and for alkaline phosphatase, a glucosylphosphatidyl-inositol-anchored protein often enriched in caveolae. Purified caveolin-enriched membrane fractions (CEMF) from bovine parathyroid cells are highly enriched in the CaR and alkaline phosphatase. Other signaling proteins, including $G_{q/11}$, ENOS, and several protein kinase C isoforms (i.e. α, δ, and ζ) are also present in CEMF. Activation of the CaR by high Ca^{2+}_o increases tyrosine phosphorylation of caveolin-1 in CEMF, suggesting that CaR-mediated signal transduction potentially involved in Ca^{2+}_o regulated processes in parathyroid cells occur in caveolae-like domains.

3.62 Palmitoylation of Neurofascin at a Site in the Membrane-Spanning Domain Highly Conserved Among the L1 Family of Cell Adhesion Molecules

Ren, Q. and Bennett, V.

J. Neurochem., ***70(5)****, 1839-1849 (1998)*

This report presents the first evidence that a member of the L1 family of nervous system cell-adhesion molecules is covalently modified by thioesterification with palmitate, and identifies a highly conserved cysteine in the predicted membrane-spanning domain as the site of modification. Neurofascin is constitutively palmitoylated at cysteine-1213 at close to a 1:1 molar stoichiometry. Kinetics of palmitate incorporation into neurofascin expressed in resting neuroblastoma cells indicate that the palmitate modification has the same turnover rate as the polypeptide chain and does not affect the protein stability of neurofascin. Palmitoylation of neurofascin expressed in dorsal root ganglion neurons is not required for delivery of neurofascin to the plasma membrane or targeting to axons. Palmitoylation also has no effect on ankyrin-binding activity of neurofascin, on the oligomeric state of neurofascin in solution, or on cell-adhesion activity of neurofascin expressed in neuroblastoma cells. A significant difference between native and C1213L neurofascin is that these proteins were localized in distinct fractions within a low-density membrane population enriched in signaling molecules. These results indicate a palmitate-dependent targeting of neurofascin to a specialized membrane microdomain.

3.63 The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions.

Sheff, D.R., Daro, E.A., Hull, M. and Mellmann, I.

J. Cell Biol., ***145(1),*** *123-139 (1999)*

Receptor recycling involves two endosome populations, peripheral early endosomes and perinuclear recycling endosomes. In polarized epithelial cells, either or both populations must be able to sort apical from basolateral proteins, returning each to its appropriate plasma membrane domain. However, neither the roles of early versus recycling endosomes in polarity nor their relationship to each other has been quantitatively evaluated. Using a combined morphological, biochemical, and kinetic approach, we found these two endosome populations to represent physically and functionally distinct compartments. Early and recycling endosomes were resolved on **Optiprep** gradients and shown to be differentially associated with rab4, rab11, and transferrin receptor; rab4 was enriched on early endosomes and at least partially depleted from recycling endosomes, with the opposite being true for rab11 and transferrin receptor. The two populations were also pharmacologically distinct, with AlF_4 selectively blocking export of transferrin receptor from recycling endosomes to the basolateral plasma membrane. We applied these observations to a detailed kinetic analysis of transferrin and dimeric IgA recycling and transcytosis. The data from these experiments permitted the construction of a testable, mathematical model which enabled a dissection of the roles of early and recycling endosomes in polarized receptor transport. Contrary to expectations, the majority (>65%) of recycling to the basolateral surface is likely to occur from early endosomes, but with relatively little sorting of apical from

basolateral proteins. Instead, more complete segregation of basolateral receptors from receptors intended for transcytosis occurred upon delivery to recycling endosomes.

3.64 Opposing effects of reactive oxygen species and cholesterol on endothelial nitric oxide synthase and endothelial cell caveolae.
Peterson, T.E. et al.
*Circulation Res., **85**, 29-37 (1999)*

Synthesis of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) is critical for normal vascular home-ostasis. eNOS function is rapidly regulated by agonists and blood flow and chronically by factors that regulate mRNA stability and gene transcription. Recently, localization of eNOS to specialized plasma membrane invagin-ations termed caveolae has been proposed to be required for maximal eNOS activity. Because caveolae are highly enriched in cholesterol, and hypercholesterolemia is associated with increased NO production, we first studied the effects of cholesterol loading on eNOS localization and NO production in cultured bovine aortic endothelial cells (BAECs). Caveolae-enriched fractions were prepared by OptiPrep gradient density centrifug-ation. Treatment of BAECs with 30 μg/mL cholesterol for 24 hours stimulated significant increases in total eNOS protein expression (1.50-fold), eNOS associated with caveolae-enriched membranes (2.23-fold), and calcium ionophore-stimulated NO production (1.56-fold). Because reactive oxygen species (ROS) contribute to endothelial dysfunction in hypercholesterolemia, we next studied the effects of ROS on eNOS localization and caveolae number. Treatment of BAECs for 24 hours with 1 μmol/L LY83583, a superoxide-generating naptho-quinolinedione, decreased caveolae number measured by electron microscopy and prevented the cholesterol-mediated increases in eNOS expression. In vitro exposure of caveolae-enriched membranes to ROS (xanthine + xanthine oxidase) dissociated caveolin more readily than eNOS from the membranes. These results show that cholesterol treatment increases eNOS expression, whereas ROS treatment decreases eNOS expression and the association of eNOS with caveolin in caveolae-enriched membranes. Our data suggest that oxidative stress modulates endothelial function by regulating caveolae formation, eNOS expression, and eNOS-caveolin interactions.

3.65 Purification and characterization of rat hippocamal CA3-dendritic spines associated with mossy fiber terminals
Kiebler, M.A., Lopez-Garcia, J.C. and Leopold, P.L.
*FEBS Lett., **445**, 80-86 (1999)*

We report a revised and improved isolation procedure for CA3-dendritic spines, most of them still in association with mossy fiber terminals resulting in a 7.5-fold enrichment over nuclei and a 29-fold enrichment over myelin. Additionally, red blood cells, medullated fibers, mitochondria and small synaptosomes were significantly depleted. We show by high resolution electron microscopy that this subcellular fraction contains numerous dendritic spines with a rich ultrastructure, e.g. an intact spine apparatus, membranous organelles, free and membrane-bound polyribosomes, endocytic structures and mitochondria. This improved experimental system will allow us to study aspects of post-synaptic functions at the biochemical and molecular level.

3.66 Separation of the intracellular secretory compartment of rat liver and isolated rat hepatocytes in a single step using self-generating gradients of iodixanol
Plonne, D., Cartwright, I., Linss, W., Dargel, R., Graham, J.M. and Higgins, J.A.*Anal. Biochem., **276(1)**, 88-96 (1999)*

A novel method is described for the separation on a single gradient of the major intracellular organelles of the secretory pathway, the Golgi, the smooth endoplasmic reticulum (ER), and the rough (ER). Total microsomes were prepared from rat liver by differential centrifugation and resuspended in 20% **iodixanol**. The microsomal suspension was then layered between a 30% iodixanol cushion and a layer of 15% iodixanol and centrifuged in a vertical rotor for 2 h. The microsomes distributed in four visible bands. The gradients were collected by upward displacement and were characterized (i) by determination of UDP galactose-galactosyl-transferase (Golgi marker) NADPH-cytochrome c reductase (ER marker) and RNA (rough endoplasmic reticulum marker); (ii) by immunoblotting for TGN38 (*trans*-Golgi marker) and GS28 (*cis*-Golgi marker) and for protein disulfide isomerase (endoplasmic reticulum lumenal marker); (iii) by determination of the lipid composition; and (iv) by electron microscopy. The results suggest that the top band (density 1.045-1.090 g/ml), which contains 68% of the galactosyltransferase activity, consists of vesicles derived from the Golgi. The second broad band in the middle of the tube (density 1.130-1.160 g/ml), which contains 54% of the NADPH-cytochrome c reductase activity, consists mainly of vesicles derived from the smooth endoplasmic reticulum, overlapped at the top by a small band of Golgi-derived lamellae. The two bands at the bottom of the tube

(density 1.130-1.160 and density 1.180-1.220 g/ml) appear to contain two subfractions of vesicles derived from the rough endoplasmic reticulum.

3.67 Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells
Lafont, F., Verkade, P., Galli, T., Wimmer, C., Louvard, D. and Simons, K.
*Proc. Natl. Acad. Sci. USA, **96**, 3734-3738 (1999)*

We have investigated the relationship between apical sorting mechanism using lipid rafts and the soluble N-ethyl maleimide-sensitive factor attachment protein receptor (SNARE) machinery, which is involved in membrane docking and fusion. We first confirmed that anti-alpha-SNAP antibodies inhibit the apical pathway in Madin-Darby canine kidney (MDCK) cells; in addition, we report that a recombinant SNAP mutant inhibits this transport step. Based on t-SNARE overexpression experiments and the effect of botulinum neurotoxin E, syntaxin 3 and SNAP-23 have been implicated in apical membrane trafficking. Here, we show in permeabilized MDCK cells that antisyntaxin 3 and anti-SNAP-23 antibodies lower surface delivery of an apical reporter protein. Moreover, using a similar approach, we show that tetanus toxin-insensitive, vesicle-associated membrane protein (TI-VAMP; also called VAMP7), a recently described apical v-SNARE, is involved. Furthermore, we show the presence of syntaxin 3 and TI-VAMP in isolated apical carriers. Polarized apical sorting has been postulated to be mediated by the clustering of apical proteins into dynamic sphingolipid-cholesterol rafts. We provide evidence that syntaxin 3 and TI-VAMP are raft-associated. These data support a raft-based mechanism for the sorting of not only apically destined cargo but also of SNAREs having functions in apical membrane-docking and fusion events.

3.68 Analysis of CD44-containing lipid rafts: Recruitment of Annexin II and stabilization by the actin cytoskeleton
Oliferenko, S., et al
*J. Cell Biol., **146(4)**, 843-854 (1999)*

CD44, the major cell surface receptor for hyaluronic acid (HA), was shown to localize to detergent-resistant cholesterol-rich microdomains, called lipid rafts, in fibroblasts and blood cells. Here, we have investigated the molecular environment of CD44 within the plane of the basolateral membrane of polarized mammary epithelial cells. We show that CD44 partitions into lipid rafts that contain annexin II at their cytoplasmic face. Both CD44 and annexin II were released from these lipid rafts by sequestration of plasma membrane cholesterol. Partition of annexin II and CD44 to the same type of lipid rafts was demonstrated by cross-linking experiments in living cells. First, when CD44 was clustered at the cell surface by anti-CD44 antibodies, annexin II was recruited into the cytoplasmic leaflet of CD44 clusters. Second, the formation of intracellular, submembranous annexin II-p11 aggregates caused by expression of a trans-dominant mutant of annexin II resulted in coclustering of CD44. Moreover, a frequent redirection of actin bundles to these clusters was observed. These basolateral CD44/annexin II-lipid raft complexes were stabilized by addition of GTPγS or phalloidin in a semipermeabilized and cholesterol depleted cell system. The low lateral mobility of CD44 in the plasma membrane, as assessed with fluorescent recovery after photobleaching (FRAP), was dependent on the presence of plasma membrane cholesterol and an intact actin cytoskeleton. Disruption of the actin cytoskeleton dramatically increased the fraction of CD44 which could be recovered from the light detergent-insoluble membrane fraction. Taken together, our data indicate that in mammary epithelial cells the vast majority of CD44 interacts with annexin II in lipid rafts in a cholesterol-dependent manner. These CD44-containing lipid microdomains interact with the underlying actin cytoskeleton.

3.69 Subcellular distribution of tuberin in cells derived from brain and liver
Yamamoto, Y. and Yeung, R.S.
*Proc. Amer. Assoc. Cancer Res., **40**, #4515 (1999)*

Mutations of TSC1 and TSC2 are responsible for the autosomal syndrome of tuberous sclerosis which predisposes carriers to the development of hamartomas and neoplasia. The TSC2 product, tuberin, possesses tumor suppressor activity and *in vitro* GAP activity towards Rap1 and Rab5. However, the physiological function of tuberin remains poorly understood. Primary sequence analysis suggested a putative transmembrane domain consistent with its abundance in the P100 post nuclear fraction and indirect immunofluorescence demonstrated co-localization with Rap1 and other Golgi markers. In light of the potential role of tuberin in the vesicular trafficking, we studied its subcellular distribution in cells of primary tissues using biochemical fractionation. Microsomal fractions of rat brain and liver were separated on a continuous Iodixanol gradient and analyzed for protein expression of tuberin, rabaptin-5, a TSC2

associated protein, EEA1, and endosomal protein, and TGN38, a trans-Golgi marker. The pattern of expression was similar between tuberin, rabaptin-5 and EEA1 but distinct from that of TGN38. Further, a substantial fraction of tuberin was found free in the cytosol and was re-distributed to the supernatant under conditions of high pH. These findings suggest that tuberin may shuttle between the cytosol and membranous organelles. Further, tuberin localization is not restricted to the Golgi but includes the endosomal compartment. This is consistent with a role of tuberin in vesicular transport.

3.70 Spatial organization of EGF receptor transmodulation by PDGF

Liu, P. and Anderson, R.G.W.
Biochem. Biophys. Res. Commun., ***261****, 695-700 (1999)*

Even though the modulation of EGF receptors by PDGF is well documented, it is not known where on the cell surface cross-talk between the two receptor systems takes place. The recent finding that both populations of receptors are concentrated in cell surface caveolae suggests that the confinement of the two' receptors to this space might facilitate their interaction. Here we show that stimulation of PDGF receptors in caveolae with PDGF causes a subpopulation of EGF receptors in the same membrane fraction to become phosphorylated on tyrosine. Coincident with tyrosine phosphorylation, the binding of EGF to its receptor markedly declines. Loss of EGF binding is partially blocked by tyrosine kinase inhibitors. Despite the close proximity of the two receptors in caveolae, we saw no evidence that EGF could stimulate PDGFR tyrosine phosphorylation. These results suggest that these two receptor systems are highly organized in caveolae.

3.71 Co-expression of scavenger receptor-BI and caveolin-1 is associated with enhanced selective cholesterol ester uptake in THP-1 macrophages

Matveev, S., van der Westhuyzen, D.R. and Smart, E.J**.**
J. Lipid Res., ***40****, 1647-1654 (1999)*

Scavenger receptor (SR)-BI mediates the selective uptake of high density lipoprotein (HDL) cholesteryl esters and the efflux of free cholesterol. In Chinese hamster ovary (CHO) cells, SR-BI is predominantly associated with caveolae which we have recently demonstrated are the initial loci for membrane transfer of HDL cholesteryl esters. Because cholesterol accumulation in macrophages is a critical event in atherogenesis, we investigated the expression of SR-BI and caveolin-1 in several macrophage cell lines. Human THP-1 monocytes were examined before and after differentiation to macrophages by treatment with 200 nM phorbol ester for 72 h. Undifferentiated THP-1 cells expressed caveolin-1 weakly whereas differentiation upregulated caveolin-1 expression greater than 50-fold. In contrast, both undifferentiated and differentiated THP-1 cells expressed similar levels of SR-BI. Differentiation of THP-1 cells increased the percent of membrane cholesterol associated with caveolae from 12% ± 1.9% to 38% ± 3.1%. The increase in caveolin-1 expression was associated with a 2- to 3-fold increase in selective cholesterol ether uptake from HDL. Two mouse macrophage cell lines, J774 and RAW, expressed levels of SR-BI similar to differentiated THP-1 cells but did not express detectable levels of caveolin-1. In comparison to differentiated THP-1 cells, RAW and J774 cells internalized 9- to 10-fold less cholesteryl ester. We conclude that differentiated THP-1 cells express both caveolin-1 and SR-BI and that their co-expression is associated with enhanced selective cholesteryl ester uptake.

3.72 The class B, type I scavenger receptor promotes the selective uptake of high density lipoprotein cholesterol ethers into caveolae

Graf, G.G., Connell, P.M., van der Westhuyzen, D.R. and Smart, E.J.
J. Biol. Chem., ***274(17)****, 12043-12048 (1999)*

The uptake of cholesterol esters from high density lipoproteins (HDLS) is characterized by the initial movement of cholesterol esters into a reversible plasma membrane pool. Cholesterol esters are subsequently internalized to a nonreversible pool. Unlike the uptake of cholesterol from low density lipoproteins, cholesterol ester uptake from HDL does not involve the internalization and degradation of the particle and is therefore termed selective. The class B, type I scavenger receptor (SR-BI) has been identified as an HDL receptor and shown to mediate selective cholesterol ester uptake. SR-BI is localized to cholesterol- and sphingomyelin- rich microdomains called caveolae. Caveolae are directly involved in cholesterol trafficking. Therefore, we tested the hypothesis that caveolae are acceptors for HDL-derived cholesterol ether (CE). Our studies demonstrate that in Chinese hamster ovary cells expressing SR-BI, >80% of the plasma membrane associated CE is present in caveolae after 7.5 min of selective cholesterol ether uptake. We also show that excess, unlabeled HDL can extract the radiolabeled CE from caveolae,

demonstrating that caveolae constitute a reversible plasma membrane pool of CE. Furthermore, 50% of the caveolae-associated CE can be chased into a nonreversible pool. We conclude that caveolae are acceptors for HDL-derived cholesterol ethers, and that caveolae constitute a reversible, plasma membrane pool of cholesterol ethers.

3.73 Polarized distribution of endogenous Racl and RhoA at the cell surface

Michaely, P.A., Mineo, C. Ying, Y-S. and Anderson, R.G.W.
*J. Biol. Chem., **274(30)**, 21430-21436 (1999)*

Racl and RhoA regulate membrane ruffling and stress fiber formation. Both molecules appear to exert their control from the plasma membrane. In fibroblasts stimulated with platelet-derived growth factor or lysophosphatidic acid, the reorganization of the cytoskeleton begins at specific sites on the cell surface. We now report that endogenous Racl and RhoA also have a polarized distribution at the cell surface. Cell fractionation and immunogold labeling show that in quiescent fibroblasts both of these molecules are concentrated in caveolae, which are plasma membrane domains that are associated with actin-rich regions of the cell. Treatment of these cells with platelet-derived growth factor stimulated the recruitment of additional Racl and RhoA to caveolae fractions, while lysophosphatidic acid only caused the recruitment of RhoA. We could reconstitute the recruitment of RhoA using either whole cell lysates or purified caveolae. Surprisingly, pretreatment of the lysates with exoenzyme C3 shifted both resident and recruited RhoA from caveolae to noncaveolae membranes. The shift in location was not caused by inactivation of the RhoA effector domain. Moreover, chimeric proteins containing the C-terminal consensus site for Racl and RhoA prenylation were constitutively targeted to caveolae fractions. These results suggest that the polarized distribution of Rho family proteins at the cell surface involves an initial targeting of the protein to caveolae and a mechanism for retaining it at this site.

3.74 Regulated migration of epidermal growth factor receptor from caveolae

Mineo, C., Gill, G.N. and Anderson, R.G.W.
*J. Biol. Chem., **274(43)**, 30636-30643 (1999)*

In quiescent fibroblasts, epidermal growth factor (EGF) receptors (EGFR) are initially concentrated in caveolae but rapidly move out of this membrane domain in response to EGF. To better understand the dynamic localization of EGFR to caveolae, we have studied the behavior of wild-type and mutant receptors expressed in cells lacking endogenous EGFR. All of the receptors we examined, including those missing the first 274 amino acids or most of the cytoplasmic tail, were constitutively concentrated in caveolae. By contrast, migration from caveolae required EGF binding, an active receptor kinase domain, and at least one of the five tyrosine residues present in the regulatory domain of the receptor. Movement appears to be modulated by Src kinase, is blocked by activators of protein kinase C, and occurs independently of internalization by clathrin coated pits. Two mutant receptors previously shown to induce an oncogenic phenotype lack the ability to move from caveolae in response to EGF, suggesting that a prolonged residence in this domain may contribute to abnormal cell behavior.

3.75 Oxidized low density lipoprotein displaces endothelial nitric-oxide synthase (eNOS) from plasmalemmal caveolae and impairs eNOS activation

Blair, A., Shaul, P.W., Yuhanna, I.S., Conrad, P.A. and Smart, E.J.
*J. Biol Chem., **274(45)**, 32512-32519 (1999)*

Hypercholesterolemia-induced vascular disease and atherosclerosis are characterized by a decrease in the bioavailability of endothelium-derived nitric oxide. Endothelial nitric-oxide synthase (eNOS) associates with caveolae and is directly regulated by the caveolar protein, caveolin. In the present study, we examined the effects of oxidized low density lipoprotein (oxLDL) on the subcellular location of eNOS, on eNOS activation, and on caveolar cholesterol in endothelial cells: We found that treatment with 10 μg/ml oxLDL for 60 min causes greater than 90% of eNOS and caveolin to leave caveolae. Treatment with oxLDL also inhibited acetylcholine-induced activation of eNOS but not prostacyclin production. oxLDL did not affect total cellular eNOS abundance. Oxidized LDL also did not affect the palmitoylation, myristoylation or phosphorylation of eNOS. Oxidized LDL, but not native LDL, or HDL depleted caveolae of cholesterol by serving as an acceptor for cholesterol. Cyclodextrin also depleted caveolae of cholesterol and caused eNOS and caveolin to translocate from caveolae. Furthermore, removal of oxLDL allowed eNOS and caveolin to return to caveolae. We conclude that oxLDL-induced depletion of caveolar cholesterol causes eNOS to leave caveolae and inhibits acetylcholine-induced activation of the enzyme. This process may be an important mechanism in the early pathogenesis of atherosclerosis.

3.76 Subcellular location of enzyme involved in oxidation on *n*-alkane by *Cladosporium resinae*
Goswami, P. and Cooney, J.J.
*Appl. Microbiol. Biotechnol., **51(6)**, 860-864 (1999)*

More than 70% of *n*-hexadecane-grown cells of *Cladosporium resinae* ATCC 22711 were converted to spheroplasts when they were treated with chinitase and lytic enzyme from *Trichoderma harziamum.* The light mitochondrial fraction, containing microbodies, mitochondria and vacuoles, was isolated from spheroplasts. Vacuoles in cells were demonstrated by the inability of acridine orange to stain organelles previously treated with 2.5 μM Bafilomycin A_1, a vacuolar ATPase inhibitor. Microbodies, mitochondria and vacuoles were separated from the light mitochondrial fraction by self-generated density gradient ultracentrifugation using **iodixanol** as gradient medium. NADH-dependent *n*-alkane monooxygenase activity and fatty alcohol oxidase activity were located in the cytoplasm and mitochondrial fractions respectively.

3.77 Persistent membrane association of activated and depalmitoylated G protein α subunits
Huang, C., Duncan, J.A., Gilman, A.G. and Mumby, S.M.
*Proc. Natl. Acad. Sci. USA, **96**, 412-417 (1999)*

Heterotrimeric signal-transducing G proteins are organized at the inner surface of the plasma membrane, where they are positioned to interact with membrane-spanning receptors and appropriate effectors. G proteins are activated when they bind GTP and inactivated when they hydrolyze the nucleotide to GDP. However, the topological fate of activated G protein α subunits is disputed. One model declares that depalmitoylation of α, which accompanies activation by a receptor, promotes release of the protein into the cytoplasm. Our data suggest that activation of G protein α subunits causes them to concentrate in subdomains of the plasma membrane but not to be released from the membrane. Furthermore, α subunits remained bound to the membrane when they were activated with guanosine5'-(3-*0*-thio)triphosphate and depalmitoylated with an acyl protein thioesterase. Limitation of α subunits to the plasma membrane obviously restricts their mobility and may contribute to the efficiency and specificity of signaling.

3.78 The yeast frataxin homologue mediates mitochondrial iron efflux
Radisky, D.C., Babcock, M.C. and Kaplan, J.
*J. Biol. Chem.,**274(8)**, 4497-4499 (1999)*

Mutations in the nuclear gene encoding the mitochondrial protein frataxin are responsible for the neurological disorder Friedreich ataxia (FA). Yeast strains with a deletion in the frataxin homologue *YFH1* accumulate excess iron in mitochondria and demonstrate mitochondrial damage. We show that in the absence of *YFH1*, mitochondrial damage is proportional to the concentration and duration of exposure to extracellular iron, establishing mitochondrial iron accumulation as causal to mitochondrial damage. Reintroduction of *YFH1* results in the rapid export of accumulated mitochondrial iron into the cytosol as free, non-heme bound iron, demonstrating that mitochondrial iron in the yeast FA model can be made bioavailable. These results demonstrate a mitochondrial iron cycle in which Yfhlp regulates mitochondrial iron efflux.

3.79 Induction of caveolae in the apical plasma membrane of Madin-Darby canine kidney cells
Verkade, P., Harder, T., Lafont, F. And Simons, K.
*J. Cell Biol., **148(4)**, 727-739 (1999)*

In this paper, we have analyzed the behavior of antibody cross-linked raft-associated proteins on the surface of MDCK cells. We observed that cross-linking of membrane proteins gave different results depending on whether cross-linking occurred on the apical or basolateral plasma membrane. Whereas antibody cross-linking induced the formation of large clusters on the basolateral membrane, resembling those observed on the surface of fibroblasts (Harder, T, P. Scheiffele, P. Verkade, and K. Simons. 1998. *J Cell Biol*. 929-942), only small (~100 nm) clusters formed on the apical plasma membrane. Cross-linked apical raft proteins e.g., GPI-anchored placental alkaline phosphatase (PLAP), influenza hemagglutinin, and gpl14 coclustered and were internalized slowly (~10% after 60 min). Endocytosis occurred through surface invaginations that corresponded in size to caveolae and were labeled with caveolin-1 antibodies. Upon cholesterol depletion the internalization of PLAP was completely inhibited. In contrast, when a non-raft protein, the mutant LDL receptor LDLR-CT22, was cross-linked, it was excluded from the clusters of raft proteins and was rapidly internalized via clathrin-coated pits. Since caveolae are normally present on

the basolateral membrane but lacking from the apical side, our data demonstrate that antibody cross-linking induced the formation of caveolae, which slowly internalized cross-linked clusters of raft-associated proteins.

3.80 Regulation of β-amyloid secretion by FE65, an amyloid protein precursor-binding protein
Sabo, S.L. et al
J. Biol. Chem., ***274(12)***, *7952-7957 (1999)*

The principal component of Alzheimer's amyloid plaques, Aβ, derives from proteolytic processing of the Alzheimer's amyloid protein precursor (APP). FE65 is a brain-enriched protein that binds to APP. Although several laboratories have characterized the APP-FE65 interaction in *vitro,* the possible relevance of this interaction to Alzheimer's disease has remained unclear. We demonstrate here that APP and FE65 co-localize in the endoplasmic reticulum/Golgi and possibly in endosomes. Moreover, FE65 increases translocation of APP to the cell surface, as well as both αAPP_s and Aβ secretion. The dramatic (4-fold) FE65-dependent increase in Aβ secretion suggests that agents which inhibit the interaction of FE65 with APP might reduce Aβ secretion in the brain and therefore be useful for preventing or slowing amyloid plaque formation.

3.81 The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate
Merritt, S.E. et al
J. Biol. Chem., ***274(15)***, *10195-10202 (1999)*

Mixed lineage kinases DLK (dual leucine zipper-bearing kinase) and MLK3 have been proposed to function as mitogen-activated protein kinase kinase kinases in pathways leading to stress-activated protein kinase/c-Jun NH_2-terminal kinase activation. Differences in primary protein structure place these MLK (mixed lineage kinase) enzymes in separate subfamilies and suggest that they perform distinct functional roles. Both DLK and MLK3 associated with, phosphorylated, and activated MKK7 in *vitro.* Unlike MLK3, however, DLK did not phosphorylate or activate recombinant MKK4 *in vitro.* In confirmatory experiments performed *in vivo,* DLK both associated with and activated MKK7. The relative localization of endogenous DLK, MLK3, MKK4, and MKK7 was determined in cells of the nervous system. Distinct from MLK3, which was identified in non-neuronal cells, DLK and MKK7 were detected predominantly in neurons in sections of adult rat cortex by immunocytochemistry. Subcellular fractionation experiments of cerebral cortex identified DLK and MKK7 in similar nuclear and extranuclear subcellular compartments. Concordant with biochemical experiments, however, MKK4 occupied compartments distinct from that of DLK and MKK7. That DLK and MKK7 occupied subcellular compartments distinct from MKK4 was confirmed by immunocytochemistry in primary neuronal culture. The dissimilar cellular specificity of DLK and MLK3 and the specific substrate utilization and subcellular compartmentation of DLK suggest that specific mixed lineage kinases participate in unique signal transduction events.

3.82 Mgm101p is a novel component of the mitochondrial nucleoid that binds DNA and is required for the repair of oxidatively damaged mitochondrial DNA
Meeusen, S. et al
J. Cell Biol., ***145(2)***, *291-304 (1999)*

Maintenance of mitochondrial DNA (mtDNA) during cell division is required for progeny to be respiratory competent. Maintenance involves the replication, repair, assembly, segregation, and partitioning of the mitochondrial nucleoid. *MGM101* has been identified as a gene essential for mtDNA maintenance in *S. cerevisiae,* but its role is unknown. Using liquid chromatography coupled with tandem mass spectrometry, we identified Mgm101p as a component of highly enriched nucleoids, suggesting that it plays a nucleoid specific role in maintenance. Subcellular fractionation, indirect immunofluorescence and GFP tagging show that Mgm101p is exclusively associated with the mitochondrial nucleoid structure in cells. Furthermore, DNA affinity chromatography of nucleoid extracts indicates that Mgm101p binds to DNA, suggesting that its nucleoid localization is in part due to this activity. Phenotypic analysis of cells containing a temperature sensi*tive mgm101* allele suggests that Mgml0lp is not involved in mtDNA packaging, segregation, partitioning or required for ongoing mtDNA replication. We examined Mgml0lp's role in mtDNA repair. As compared with wild-type cells, *mgm101* cells were more sensitive to mtDNA damage induced by UV irradiation and were hypersensitive to mtDNA damage induced by gamma rays and H_2O_2 treatment. Thus, we propose that Mgm101p performs an essential function in the repair of oxidatively damaged mtDNA that is required for the maintenance of the mitochondrial genome.

3.83 Association of sterol- and glycosylphosphatidylinositol-linked proteins with *Drosophilia* raft lipid microdomains

Rietveld, A., Neutz, S., Simons, K. and Eaton, S.
J. Biol. Chem., ***274(17)***, *12049-12054 (1999)*

In vertebrates, the formation of raft lipid microdomains plays an important part in both polarized protein sorting and signal transduction. To establish a system in which raft-dependent processes could be studied genetically, we have analyzed the protein and lipid composition of these microdomains in *Drosophila melanogaster*. Using mass spectrometry, we identified the phospholipids, sphingolipids, and sterols present in *Drosophila* membranes. Despite chemical differences between *Drosophila* and mammalian lipids, their structure suggests that the biophysical properties that allow raft formation have been preserved. Consistent with this, we have identified a detergent-insoluble fraction of *Drosophila* membranes that, like mammalian rafts, is rich in sterol, sphingolipids, and glycosylphosphatidylinositol-linked proteins. We show that the sterol-linked Hedgehog N-terminal fragment associates specifically with this detergent-insoluble membrane fraction. Our findings demonstrate that raft formation is preserved across widely separated phyla in organisms with different lipid structures. They further suggest sterol modification as a novel mechanism for targeting proteins to raft membranes and raise the possibility that signaling and polarized intracellular transport of Hedgehog are based on raft association.

3.84 N-glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells

Benting, J.H., Rietveld, A.G. and Simons, K.
J. Cell Biol., ***146(2)***, *313-320 (1999)*

Glycosyl-phosphatidylinositol (GPI)-anchored proteins are preferentially transported to the apical cell surface of polarized Madin-Darby canine kidney (MDCK) cells. It has been assumed that the GPI anchor itself acts as an apical determinant by its interaction with sphingolipid-cholesterol rafts. We modified the rat growth hormone (rGH), an unglycosylated, unpolarized secreted protein, into a GPI-anchored protein and analyzed its surface delivery in polarized MDCK cells. The addition of a GPI anchor to rGH did not lead to an increase in apical delivery of the protein. However, addition of N-glycans to GPI-anchored rGH resulted in predominant apical delivery, suggesting that N-glycans act as apical sorting signals on GPI-anchored proteins as they do on transmembrane and secretary proteins, In contrast to the GPI-anchored rGH, a transmembrane form of rGH which was not raft-associated accumulated intracellularly. Addition of N-glycans to this chimeric protein prevented intracellular accumulation and led to apical delivery.

3.85 Identification and characterization of polycystin-2, the *PKD2* gene product

Cai, Y. et al
J. Biol. Chem., ***274(40)***, *28557-28565 (1999)*

PKD2, the second gene for the autosomal dominant polycystic kidney disease (ADPKD), encodes a protein, polycystin-2, with predicted structural similarity to cation channel subunits. However, the function of polycystin-2 remains unknown. We used polyclonal antisera specific for the intracellular NH_2 and COOH termini to identify polycystin-2 as an ~110-kDa integral membrane glycoprotein. Polycystin-2 from both native tissues and cells in culture is sensitive to Endo H suggesting the continued presence of high-mannose oligosaccharides typical of pre-middle Golgi proteins. Immunofluorescent cell staining of polycystin-2 shows a pattern consistent with localization in the endoplasmic reticulum. This finding is confirmed by co-localization with protein-disulfide isomerase as determined by double indirect immunofluorescence and co-distribution with calnexin in subcellular fractionation studies. Polycystin-2 translation products truncated at or after Gly^{821} retain their exclusive endoplasmic reticulum localization while products truncated at or before Glu^{787} additionally traffic to the plasma membrane. Truncation mutants that traffic to the plasma membrane acquire Endo H resistance and can be biotinylated on the cell surface in intact cells. The 34-amino acid region GLU^{787}-Ser^{820}, containing two putative phosphorylation sites, is responsible for the exclusive endoplasmic reticulum localization of polycystin-2 and is the site of specific interaction with an as yet unidentified protein binding partner for polycystin-2. The localization of full-length polycystin-2 to intracellular membranes raises the possibility that the PKD2 gene product is a subunit of intracellular channel complexes.

3.86 NK lytic-associated molecule: A novel gene selectively expressed in cells with cytolytic function
Kozlowski, M., Schorey, J., Portis, T., Grigoriev, V. and Kornbluth, J.
*J. Immunol., **163**, 1775-1785 (1999)*

NK cells are most effective in killing a broad spectrum of primary tumor cells after stimulation with cytokines. We have cloned a novel gene, designated NKLAM (for NK lytic-associated molecule), whose expression is associated with this cytokine-enhanced process. NKLAM expression is up-regulated in NK cells by IL-2 and IFNβ. NKLAM is also selectively expressed by activated macrophages and CTL. Treatment of NK cells and CTL with NKLAM antisense oligonucleotides specifically decreases their cytolytic activity, while having no effect on cell growth. The NKLAM gene encodes a 62-kDa ring finger-containing protein that localizes to the cytoplasmic granules in NK cells. Further study of this gene may add to our understanding of cytotoxic processes common to NK cells, CTL, and activated macrophages.

3.87 Glycoprotein reglucosylation and nucleotide sugar utilization in the secretory pathway: identification of a nucleoside diphosphatase in the endoplasmic reticulum
Trombetta, E.S. and Helenius, A.
*The EMBO J., **18(12)**, 3282-3292 (1999)*

UDP is generated in the lumen of the endoplasmic reticulum (ER) as a product of the UDP-glucose-dependent glycoprotein reglucosylation in the calnexin/calreticulin cycle. We describe here the identification, purification and characterization of an ER enzyme that hydrolyzes UDP to UMP. This nucleoside diphosphatase is a ubiquitously expressed, soluble 45 kDa glycoprotein devoid of transmembrane domains and KDEL-related ER localization sequences. It requires divalent cations for activity and hydrolyzes UDP, GDP and IDP but not any other nucleoside di-, mono- or triphosphates, nor thiamine pyrophosphate. By eliminating UDP, which is an inhibitory product of the UDP-Glc:glycoprotein glucosyltransferase, it is likely to promote reglucosylation reactions involved in glycoprotein folding and quality control in the ER.

3.88 Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator
Bradbury, N.A. et al
*Am. J. Physiol., **276** (Lung Cell. Mol. Physiol., 20), L659-L668 (1999)*

Mutations in the gene encoding the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) chloride channel give rise to the most common lethal genetic disease of Caucasian populations, CF. Although the function of CFTR is primarily related to the regulation of apical membrane chloride permeability, biochemical, immunocytochemical, and functional studies indicate that CFTR is also present in endosomal and *trans* Golgi compartments. The molecular pathways by which CFTR is internalized into intracellular compartments are not fully understood. To define the pathways for CFTR internalization, we investigated the association of CFTR with two specialized domains of the plasma membrane, clathrin-coated pits and caveolae. Internalization of CFTR was monitored after cell surface biotinylation and quantitation of cell surface CFTR levels after elution of cell lysates from a monomeric avidin column. Cell surface levels of CFTR were determined after disruption of caveolae or clathrin-coated vesicle formation. Biochemical assays revealed that disrupting the formation of clathrin-coated vesicles inhibited the internalization of CFTR from the plasma membrane, resulting in a threefold increase in the steady-state levels of cell surface CFTR. In contrast, the levels of cell surface CFTR after disruption of caveolae were not different from those in control cells. In addition, although our studies show the presence of caveolin at the apical membrane domain of human airway epithelial cells, we were unable to detect CFTR in purified caveolae. These results suggest that CFTR is constitutively internalized from the apical plasma membrane via clathrin-coated pits and that CFTR is excluded from caveolae.

3.89 Cloning, expression, and cellular localization of a human prenylcysteine lyase
Tschantz, W.R., Zhang, L. and Casey, P.J
*J. Biol. Chem., **274(50)**, 35802-35808 (1999)*

Prenylated proteins contain either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid covalently attached to cysteine residues at or near their C terminus. These proteins constitute up to 2% of total cellular protein in eukaryotic cells. The degradation of prenylated proteins raises a metabolic challenge to the cell, because the thioether bond of the modified cysteine is quite stable. We recently identified and isolated an enzyme termed prenylcysteine lyase that cleaves the prenylcysteine to free cysteine and an

isoprenoid product (Zhang, L., Tschantz, W. IL, and Casey, P. J. (1997) *J. Biol Chem.* 272,23354-23359). To facilitate the molecular characterization of this enzyme, its cloning was undertaken. Overlapping cDNA clones encoding the complete coding sequence of this enzyme were obtained from a human cDNA library. The open reading frame of the gene encoding prenylcysteine lyase is 1515 base pairs and has a nearly ubiquitous expression pattern with a message size of 6 kilobase pairs. Recombinant prenylcysteine lyase was produced in a baculovirus-Sf9 expression system. Analysis of both the recombinant and native enzyme revealed that the enzyme is glycosylated and contains a signal peptide that is cleaved during processing. Additionally, the subcellular localization of this enzyme was determined to be lysosomal. These findings strengthen the notion that prenylcysteine lyase plays an important role in the final step in the degradation of prenylated proteins and will allow further physiological and biochemical characterization of this enzyme.

3.90 The amyloid precursor protein interacts with G_o heterotrimeric protein within a cell compartment specialized in signal transduction

Brouillett, E. et al
J. Neurosci., ***19(5)***, *1717-1727 (1999)*

The function of the β-amyloid protein precursor (βAPP), a transmembrane molecule involved in Alzheimer pathologies, is poorly understood. We recently reported the presence of a fraction of βAPP in cholesterol and sphingoglycolipid-enriched microdomains (CSEM), a caveolae-like compartment specialized in signal transduction. To investigate whether βAPP actually interferes with cell signaling, we reexamined the interaction between βAPP and G_o GTPase. In strong contrast with results obtained with reconstituted phospholipid vesicles (Okamoto et al., 1995), we find that incubating total neuronal membranes with 22Cl1, an antibody that recognizes an N-terminal βAPP epitope, reduces high-affinity G_o GTPase activity. This inhibition is specific of $G_{\alpha o}$ and is reproduced, in the absence of 22Cl1, by the addition of the βAPP C-terminal domain but not by two distinct mutated βAPP C-terminal domains that do not bind $G_{\alpha o}$. This inhibition of $G_{\alpha o}$ GTPase activity by either 22Cl1 or wild-type βAPP cytoplasmic domain suggests that intracellular interactions between βAPP and $G_{\alpha o}$ could be regulated by extracellular signals. To verify whether this interaction is preserved in CSEM, we first used biochemical, immunocytochemical, and ultrastructural techniques to unambiguously confirm the colocalization of $G_{\alpha o}$ and βAPP in CSEM. We show that inhibition of basal $G_{\alpha o}$ GTPase activity also occurs within CSEM and correlates with the coimmunoprecipitation of $G_{\alpha o}$ and βAPP. The regulation of $G_{\alpha o}$ GTPase activity by βAPP in a compartment specialized in signaling may have important consequences for our understanding of the physiopathological functions of βAPP.

3.91 ER/Golgi intermediates acquire Golgi enzymes by Bredelfin A – sensitive retrograde transport in vitro

Lin, C-C., Love, H.D., Gushue, J.N., Bergeron, J.J.M. and Osterman, J.
J. Cell. Biol., ***147(7)***, *1457-1472 (1999)*

Secretary proteins exit the ER in transport vesicles that fuse to form vesicular tubular clusters (VTCs) which move along microtubule tracks to the Golgi apparatus. Using the well-characterized in vitro approach to study the properties of Golgi membranes, we determined whether the Golgi enzyme NAGT I is transported to ER/Golgi intermediates. Secretary cargo was arrested at distinct steps of the secretary pathway of a glycosylation mutant cell line, and in vitro complementation of the glycosylation defect was determined. Complementation yield increased after ER exit of secretary cargo and was optimal when transport was blocked at an ER/Golgi intermediate step. The rapid drop of the complementation yield as secretary cargo progresses into the stack suggests that Golgi enzymes are preferentially targeted to ER/Golgi intermediates and not to membranes of the Golgi stack. Two mechanisms for in vitro complementation could be distinguished due to their different sensitivities to brefeldin A (BFA). Transport occurred either by direct fusion of preexisting transport intermediates with ER/Golgi intermediates, or it occurred as a BFA-sensitive and most likely COP I-mediated step. Direct fusion of ER/Golgi intermediates with cisternal membranes of the Golgi stack was not observed under these conditions.

3.92 Membrane raft microdomains mediate front-rear polarity in migrating cells
Manes, S. et al
*EMBO J., **18(22)**, 6211-6220 (1999)*

The acquisition of spatial and functional asymmetry between the rear and the front of the cell is a necessary step for cell chemotaxis. Insulin-like growth factor-1 (IGF-I) stimulation of the human adenocarcinoma MCF-7 induces a polarized phenotype characterized by asymmetrical CCR5 chemokine receptor redistri-bution to the leading cell edge. CCR5 associates with membrane raft microdomains, and its polarization parallels redistribution of raft molecules, including the raft-associated ganglioside GM1, glycosylphosphat-idylinositol- anchored green fluorescent protein and ephrinBl, to the leading edge. The non-raft proteins transferrin receptor and a mutant ephrinB1 are distributed homogeneously in migrating MCF-7 cells, supporting the raft localization requirement for polarization. IGF-I stimulation of cholesterol-depleted cells induces projection of multiple pseudopodia over the entire cell periphery, indicating that raft disruption specifically affects the acquisition of cell polarity but not IGF-I-induced protrusion activity. Cholesterol depletion inhibits MCF-7 chemotaxis, which is restored by replenishing cholesterol. Our results indicate that initial segregation between raft and non-raft membrane proteins mediates the necessary, redistribution of specialized molecules for cell migration.

3.93 Isolated rabbit enterocytes as a model cell system for investigations of chylomicron assembly and secretion
Cartwright, I.J. and Higgins, J.A.
*J. Lipid Res., **40**, 1357-1365 (1999)*

A method is described for the isolation of viable enterocytes from rabbit small intestine. The procedure can also be used to isolate populations of epithelial cells from the crypt/villus gradient. The isolated enterocytes synthesized and secreted apoB-48 and triacylglycerol in particles of the density of chylomicrons. Secretion was stimulated by addition of bile salt/lipid micelles. Pulse-chase experiments demonstrated that newly synthesized apoB-48 is degraded intracellularly and that degradation is inhibited by provision of lipid micelles, suggesting that regulation of chylomicron assembly and secretion is broadly similar to that of very low density lipoprotein assembly in hepatocytes. This procedure for preparation of isolated enterocytes will provide a useful model system for investigation of the molecular details of chylomicron assembly.

3.94 Caveolin-3 upregulation activates β-secretase-mediated cleavage of the amyloid precursor protein in Alzheimer's disease
Nisshiyama, K. et al
*J. Neurosci., **19(15)**, 6538-6548 (1999)*

Here, we investigate the involvement of caveolins in the pathophysiology of Alzheimer's disease (AD). We show dramatic upregulation of caveolin-3 immunoreactivity in astroglial cells surrounding senile plaques in brain tissue sections from authentic AD patients and an established transgenic mouse model of AD. In addition, we find that caveolin-3 physically interacts and biochemically colocalizes with amyloid precursor protein (APP) both *in vivo and in vitro*. Interestingly, recombinant overexpression of caveolin-3 in cultured cells stimulated β-secretase-mediated processing of APP. Immunoreactivities of APP and presenilins were concomitantly increased in caveolin-3-positive astrocytes. Because the presenilins also form a physical complex with caveolin-3, caveolin-3 may provide a common platform for APP and the presenilins to associate in astrocytes. In AD, augmented expression of caveolin-3 and presenilins in reactive astrocytes may alter APP processing, leading to the overproduction of its toxic amyloid metabolites.

3.95 Immunoisolation of caveolae with high affinity antibody binding to the oligomeric caveolin cage
Oh, P. and Schnitzer, J.E.
*J. Biol. Chem, **274 (33)**, 23144-23154 (1999)*

Defining the molecular composition of caveolae is essential in establishing their molecular architecture and functions. Here, we identify a high affinity monoclonal antibody that is specific for caveolin-h and rapidly binds caveolin oligomerized around intact caveolae. We use this antibody (i) to develop a new simplified method for rapidly isolating caveolae from cell and tissue homogenates without using the silica-coating technology and (ii) to analyze various caveolae isolation techniques to understand how they work and why they yield different compositions. Caveolae are immunoisolated from rat lung plasma membrane fractions

subjected to mechanical disruption. Sonication of plasma membranes, isolated with or without silica coating, releases caveolae along with other similarly buoyant microdomains and, therefore, requires immunoisolation to purify caveolae. Shearing of silica-coated plasma membranes provides a homogeneous population of caveolae whose constituents (i) remain unchanged after immunoisolation, (ii) all fractionate bound to the immunobeads, and (iii) appear equivalent to caveolae immunoisolated after sonication. The caveolae immunoisolated from different low density fractions are quite similar in molecular composition. They contain a subset of key signaling molecules *(i.e.* G protein and endothelial nitric oxide synthase) and are markedly depleted in glycosylphosphatidylinositol-anchored proteins, β-actin, and angiotensin-converting enzyme. All caveolae isolated from the cell surface of lung microvascular endothelium *in vivo* appear to be coated with caveolin-lα. Caveolin-1β and -2 can also exist in these same caveolae. The isolation and analytical procedures as well as the time-dependent dissociation of signaling molecules from caveolae contribute to key compositional differences reported in the literature for caveolae. This new, rapid, magnetic immunoisolation procedure provides a consistent preparation for use in the molecular analysis of caveolae.

3.96 The growth-related, translationally controlled protein P23 has properties of a tubulin protein and associates transiently with microtubules during the cell cycle
Gachet, Y. et al
J. Cell Sci., ***112***, *1257-1271 (1999)*

The translationally controlled protein P23 was discovered by the early induction of its rate of synthesis after mitogenic stimulation of mouse fibroblasts. P23 is expressed in almost all mammalian tissues and it is highly conserved between animals, plants and yeast. Based on its amino acid sequence. P23 cannot be attributed to any known protein family, and its cellular function remains to be elucidated. Here, we present evidence that P23 has properties of a tubulin binding protein that associates with microtubules in a cell cycle-dependent manner. (1) P23 is a cytoplasmic protein that occurs in complexes of 100-150 kDa, and part of P23 can be immunoprecipitated from HeLa cell extracts with anti-tubulin antibodies. (2) In Immunolocalization experiments we find P23 associated with microtubules during G_1, S, G_2 and early M phase of the cell cycle. At metaphase, P23 is also bound to the mitotic spindle, and it is detached from the spindle during metaphase-anaphase transition. (3) A GST-P23 fusion protein interacts with α- and β-tubulin, and recombinant P23 binds to taxol-stabilised microtubules in vitro. The tubulin binding domain of P23 was identified by mutational analysis; it shows similarity to part of the tubulin binding domain of the microtubule-associated protein MAP-1B. (4) Overexpression of P23 results in cell growth retardation and its alterations of cell morphology. Moreover, elevation of P23 levels leads to microtubule rearrangements and to an increase in microtubule mass and stability.

3.97 VIP36 localisation to the early secretory pathway
Fullekrug, J., Scheiffele, P. and Simons, K.
J. Cell Sci., ***112***, *2813-2821 (1999)*

VIP36, an integral membrane protein previously isolated from epithelial MDCK cells, is an intracellular lectin of the secretory pathway. Overexpressed VIP36 had been localized to the Golgi complex, plasma membrane and endocytic structures suggesting post-Golgi trafficking of this molecule (Fiedler et al, 1994). Here we provide evidence that endogenous VIP36 is localized to the Golgi apparatus and the early secretory pathway of MDCK and Vero cells and propose that retention is easily saturated. High resolution confocal microscopy, shows partial overlap of VIP36 with Golgi marker proteins. Punctate cytoplasmic structures colocalize with coatomer and ERGIC-53, labeling ER-Golgi intermediate membrane structures. Cycling of VIP36 is suggested by colocalization with anterograde cargo trapped in pre-Golgi structures and modification of its N-linked carbohydrate by glycosylation enzymes of medial Golgi cisternae. Furthermore, after brefeldin A treatment VIP36 is segregated from resident Golgi proteins and codistributes with ER-Golgi recycling proteins.

3.98 Acyl and alkyl chain length of GPI-anchors is critical for raft association in vitro
Benting, J., Rietveld, A., Ansorge, I. and Simons, K
FEBS Lett., ***462***, *47-50 (1999)*

We determined the acyl and alkyl chain composition of GPI-anchors isolated from MDCK and Fischer rat thyroid (FRT) cells. Both cell lines synthesize GPI-anchors containing C16/C18 or C18/C18 saturated acyl and alkyl chains. The GPI-anchored placental alkaline phosphatase (PLAP) expressed in both cells is raft-associated and PLAP purified from FRT cells is raft-associated in vitro when reconstituted into liposomes

containing raft lipids. In contrast, the GPI-anchored variant surface glycoprotein from *Trypanosoma brucei*, which contains C14 acyl and alkyl chains, shows no significant raft association after reconstitution in vitro. These data indicate that the acyl and alkyl chain composition of GPI-anchors determines raft association.

3.99 EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains
Bruckner, K. et al
*Neuron, **22**, 511-524 (1999)*

Transmembrane ephrinB proteins have important functions during embryonic patterning as ligands for Eph receptor tyrosine kinases and presumably as signal-transducing receptor-like molecules. Consistent with "reverse" signaling, ephrinB1 is localized in sphingo-lipid/cholesterol-enriched raft microdomains, platforms for the localized concentration and activation of signaling molecules. Glutamate receptor-interacting protein (GRIP) and a highly related protein, which we have termed GRIP2, are recruited into these rafts through association with the C-terminal PDZ target site of ephrinB1. Stimulation of ephrinB1 with soluble EphB2 receptor ectodomain causes the formation of large raft patches that also contain GRIP proteins. Moreover, a GRIP-associated serine/threonine kinase activity is recruited into ephrinB1-GRIP complexes. Our findings suggest that GRIP proteins provide a scaffold for the assembly of a multiprotein signaling complex downstream of ephrinB ligands.

3.100 In vivo imaging of tumors with protease-activated near-infrared fluorescent probes
Weissleder, R., Tung, C-H., Mahmood, U. and Bogdanov, A.
*Nature Biotech., **17**, 375-378 (1999)*

We have developed a method to image tumor-associated lysosomal protease activity in a xenograft mouse model In vivo using autoquenched near-infrared fluorescence (NIRF) probes. NIRF probes were bound to a long circulating graft copolymer consisting of poly-L-lysine and methoxypolyethylene glycol succinate. Following intravenous Injection, the NIRF probe carrier accumulated in solid tumors due to its long circulation time and leakage through tumor neovasculature. Intratumoral NIRF signal was generated by lysosomal proteases in tumor cells that cleave the macromolecule, thereby releasing previously quenched fluorochrome. In vivo imaging showed a 12-fold increase in NIRF signal, allowing the detection of tumors with submillimeter-sized diameters. This strategy can be used to detect such early stage tumors in vivo and to probe for specific enzyme activity.

3.101 Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity
Wolfe, M.S., Xia, W., Ostraszewski, B.L., Diehl, T.S., Kimberly, W.T and Selkoe, D.J.
*Nature **398**, 513-517 (1999)*

Accumulation of the amyloid-β protein (Aβ) in the cerebral cortex is an early and invariant event in the pathogenesis of Alzheimer's disease. The final step in the generation of Aβ from the β-amyloid precursor protein is an apparently intramembranous proteolysis by the elusive γ-secretase(s). The most common cause of familial Alzheimer's disease is mutation of the genes encoding presenilins 1 and 2, which alters γ-secretase activity to increase the production of the highly amyloidogenic $A\beta_{42}$ isoform. Moreover, deletion of presenilin-1 in mice greatly reduces γ-secretase activity, indicating that presenilin-1 mediates most of this proteolytic event. Here we report that mutation of either of two conserved transmembrane (TM) aspartate residues in presenilin-1, Asp 257 (in TM6) and Asp 385 (in TM7), substantially reduces Aβ production and increases the amounts of the carboxy-terminal fragments of β-amyloid precursor protein that are the substrates of γ-secretase. We observed these effects in three different cell lines as well as in cell-free microsomes. Either of the Asp→ Ala mutations also prevented the normal endoproteolysis of presenilin-1 in the TM6→TM7 cytoplasmic loop. In a functional presenilin-1 variant (carrying a deletion in exon 9) that is associated with familial Alzheimer's disease and which does not require this cleavage, the Asp 385→Ala mutation still inhibited γ-secretase activity. Our results indicate that the two transmembrane aspartate residues are critical for both presenilin-I endoproteolysis and γ-secretase activity, and suggest that presenilin 1 is either a unique diaspartyl cofactor for γ-secretase or is itself γ-secretase, an autoactivated intramembranous aspartyl protease.

3.102 Evaluation of the affinity and turnover number of both hepatic mitochondrial and microsomal carnitine acyltransferases: relevance to intracellular partitioning of Acyl-CoAs
Abo-Hashema, K.A., Cake, M.H., Lukas, M.A. and Knudsen, J.
*Biochemistry, **38**, 15840-15847 (1999)*

Mitochondrial carnitine palmitoyltransferase I (CPT I) and microsomal carnitine acyltransferase I (CAT I) regulate the entry of fatty acyl moieties into their respective organelles. Thus, CPT I and CAT I occupy prominent positions in the pathways responsible for energy generation in mitochondria and the assembly of VLDL in the endoplasmic reticulum, respectively. Previous attempts to determine the intrinsic kinetic properties of CPT I and CAT I have been hampered by the occurrence of sigmoidal velocity curves. This was overcome, in this study, by the inclusion of recombinant acyl-CoA binding protein in the assay medium. For the first time, we have determined the concentrations of total functional enzyme (E(t)) by specific radiolabeling of the active site, the dissociation constants (K(d)) and the turnover numbers of CPT I and CAT I toward the CoA esters of oleic acid (C18:1) and docosahexaenoic acid (C22:6). The data show that carnitine inhibits CAT I at physiological concentrations which are not inhibitory to CPT I. Thus, carnitine concentration is likely to be a significant factor in determining the partitioning of acyl-CoAs between mitochondria and microsomes, a role which has not been previously recognized. Moreover, the finding that CAT I elicits a lower turnover toward the CoA ester of C22:6 (25 s(-)(1)) than toward that of C18:1 (111 s(-)(1)), while having similar K(d) values, suggests the use of this polyunsaturated fatty acid to inhibit VLDL biosynthesis.

3.103 The protective role of high-density lipoproteins in atherosclerosis
Mingpeng, S. and Zongli, W.
*Exp. Gerontol., **34**, 539-548 (1999)*

Serum high-density lipoprotein level is known to be correlated inversely with the incidence and mortality rates of ischemic heart disease. Although some reports pointed out that in case of hyperalphalipoproteinemia, lesions in the coronary arteries were occasionally found, it is also noticed that in very rare condition, no atheromatous lesions found even in patients with hereditary alphalipoprotein deficiency (Funke et al., 1991). However, clinical surveys have confirmed that high high-density-lipoprotein cholesterol level is favorable in preventing the development of atheroclerotic lesion and high-density lipoprotein together with apolipoprotein AI are currently considered to be the most reliable parameters in predicting the development of atherosclerosis in hyperlipidemia.

3.104 Membrane structure of caveolae and isolated caveolin-rich vesicles
Westermann, M., Leutbecher, H. and Meyer, H.W.
*Histochem. Cell Biol., **111**, 71-81 (1999)*

Caveolae are specialized invaginated domains of the plasma membrane. Using freeze-fracture electron microscopy, the shape of caveolae and the distribution of intramembrane particles (integral membrane proteins) were analyzed. The caveolar membrane is highly curved and forms flask-like invaginations with a diameter of 80-120 nm with an open porus of 30-50 nm in diameter. The fracture faces of caveolar membranes are nearly free of intramembrane particles. Protein particles in a circular arrangement surrounding the caveolar opening were found on plasma membrane fracture faces. For isolation of caveolin-enriched membrane vesicles, the method of Triton X-100 solubilization, as well as a detergent-free isolation method, was used. The caveolin-rich vesicles had an average size of between 100 and 200 nm. No striated coat could be detected on the surface of isolated caveolin-rich vesicles. Areas of clustered intramembrane particles were found frequently on membrane fracture faces of caveolin-rich vesicles. The shape of these membrane protein clusters is often ring-like with a diameter of 30-50 nm. Membrane openings were found to be present in the caveolin-rich membrane vesicles, mostly localized in the areas of the clustered membrane proteins. Immunogold labeling of caveolin showed that the protein is a component within the membrane protein clusters and is not randomly distributed on the membrane of caveolin-rich vesicles.

3.105 Caveolin interacts with Trk A and p75^{NTR} and regulates neurotrophin signaling pathways
Bilderback, T.R., Gazula, V-R., Lisanti, M.P. and Dobrowsky, R.T.
*J. Biol. Chem., **274(1)**, 257-263 (1999)*

Neurotrophins signal through Trk tyrosine kinase receptors and the low-affinity neurotrophin receptor p75^{NTR}. We have shown previously that activation of Trk A tyrosine kinase activity can inhibit p75^{NTR}-dependent sphingomyelin hydrolysis, that caveolae are a localized site for p75^{NTR} signaling, and that caveolin can directly interact with p75^{NTR}. The ability of caveolin to also interact with tyrosine kinase receptors and inhibit their activity led us to hypothesize that caveolin expression may modulate interactions between neurotrophin signaling pathways. PC12 cells were transfected with caveolin that was expressed efficiently and targeted to the appropriate membrane domains. Upon exposure to nerve growth factor (NGF), caveolin-PC12 cells were unable to develop extensive neuritic processes. Caveolin expression in PC12 cells was found to diminish the magnitude and duration of Trk A activation *in vivo*. This inhibition may be due to a direct interaction of caveolin with Trk A, because Trk A co-immunoprecipitated with caveolin from Cav-Trk A-PC12 cells, and a glutathione *S*-transferase-caveolin fusion protein bound to Trk A and inhibited NGF-induced autophosphorylation *in vitro*. Furthermore, the *in vivo* kinetics of the inhibition of Trk A tyrosine kinase activity by caveolin expression correlated with an increased ability of NGF to induce sphingomyelin hydrolysis through p75^{NTR}. In summary, our results suggest that the interaction of caveolin with neurotrophin receptors may have functional consequences in regulating signaling through p75^{NTR} and Trk A in neuronal and glial cell populations.

3.106 Presence of cholyl- and chenodexoycholyl- coenzyme A thioesterase activity in human liver
Solaas, K., Sletta, R.J., Sρreide, O. and Kase, B.F.
*Scand. J. Clin. Lab. Invest., **60**, 91-102 (2000)*

In human liver homogenate the formation of bile acid..CoA thioesters is localized both to the microsomal fraction catalysed by an ATP-dependent synthetase and to the peroxisomal fraction catalysed by the thiolase in the last step of the β-oxidative cleavage of the 5β-cholestanoyl side chain. The cytosolic bile acid-CoA:amino acid N-acyltransferase catalyse the conjugation of the CoA-activated bile acids with taurine or glycine prior to secretion into bile. The formation of bile acid-CoA esters is considered the rate-limiting step in bile acid amidation. So far, a bile acid-CoA cleaving activity has not been assessed in the research of bile acid aniidation in human liver. In this work, a bile acid-CoA cleaving activity has been demonstrated at a rate that may influence the concentration of bile acid-CoA thioesters, free bile acids and amidated bile acids within the hepatocyte. Recently, it was shown that free chenodeoxycholic acid, formed by the thioesterase, is the physiological ligand of the farnesoid X receptor.

A multiorganelle distribution of the bile acid-CoA hydrolytic activity was found. In the postnuclear fraction of human liver homogenate, apparent K_m and V_{max} for the cleavage of choloyl-CoA were 7.7 x 10^{-5} mol/L and 3.6 nmol x mg^{-1} x min^{-1}, respectively. The corresponding values for chenodeoxycholoyl-CoA cleavage were 7.1 x 10^{-5} mol/L and 4.8 nmol x mg^{-1} x min^{-1}. Hydrolytic activities were detected in the microsomal and the peroxisomal fractions where the bile acid-CoA esters are formed as well as in cytosol housing the N-acyltransferase activity. Compared to the bile acid-CoA synthetase activities, the hydrolytic activities were considerably higher, both in the postnuclear fraction and in the microsomal fraction. The thioesterase activities were in the same range as detected for the N-acyltransferase activities both in the postnuclear fraction and in the cytosolic fraction. The mere presence of thioesterase in microsomes, peroxisomes and cytosol seems counterproductive to bile acid amidation. The thioesterases may have an indirect regulatory function on the bile acid synthesis and are important for the regulation of bile acid synthesis by providing free chenodeoxycholic acid, the most potent activator of the farnesoid X receptor.

3.107 Characterization of insulin-responsive GLUT4 storage vesicles isolated from 3T3-L1 adipocytes
Hashiramoto, M. and James, D.E.
*Mol. Cell Biol., **20(1)**, 416-427 (2000)*

Insulin regulates glucose transport in muscle and adipose tissue by triggering translocation of a facilitative glucose transporter, GLUT4, from an intracellular compartment to the cell surface. It has previously been suggested that GLUT4 is segregated between endosomes, the trans-Golgi network (TGN), and a postendosomal storage compartment. The aim of the present study was to isolate the GLUT4 storage compartment in order to determine the relationship of this compartment to other organelles, its components, and its presence in different cell types. A crude intracellular membrane fraction was prepared from 3T3-L1 adipocytes and subjected to **iodixanol** equilibrium sedimentation analysis. Two distinct GLUT4-containing

vesicle peaks were resolved by this procedure. The lighter of the two peaks (peak 2) was comprised of two overlapping peaks: peak 2b contained recycling endosomal markers such as the transferrin receptor (TfR), cellubrevin, and Rab4, and peak 2a was enriched in TGN markers (syntaxin 6, the cation-dependent mannose 6-phosphate receptor, sortilin, and sialyltransferase). Peak 1 contained a significant proportion of GLUT4 with a smaller but significant amount of cellubrevin and relatively little TfR. In agreement with these data, internalized transferrin (Tf) accumulated in peak 2 but not peak 1. There was a quantitatively greater loss of GLUT4 from peak 1 than from peak 2 in response to insulin stimulation. These data, combined with the observation that GLUT4 became more sensitive to ablation with Tf-horseradish peroxidase following insulin treatment, suggest that the vesicles enriched in peak 1 are highly insulin responsive. Iodixanol gradient analysis of membranes isolated from other cell types indicated that a substantial proportion of GLUT4 was targeted to peak 1 in skeletal muscle, whereas in CHO cells most of the GLUT4 was targeted to peak 2. These results indicate that in insulin-sensitive cells GLUT4 is targeted to a subpopulation of vesicles that appear, based on their protein composition, to be a derivative of the endosome. We suggest that the biogenesis of this compartment may mediate withdrawal of GLUT4 from the recycling system and provide the basis for the marked insulin responsiveness of GLUT4 that is unique to muscle and adipocytes.

3.108 Activated cardiac adenosine A_1 receptors translocate out of caveolae

Lasley, R.D., Narayan, P., Uittenbogaard, A. and Smart, E.J.
J. Biol. Chem., ***275(6)***, *4417-4421 (2000)*

The cardiac affects of the purine nucleoside, adenosine, are well known. Adenosine increases coronary blood flow, exerts direct negative chronotropic and dromotropic effects, and exerts indirect anti-adrenergic effects. These effects of adenosine are mediated via the activation of specific G protein-coupled receptors. There is increasing evidence that caveolae play a role in the compartmentalization of receptors and second messengers in the vicinity of the plasma membrane. Several reports demonstrate that G protein-coupled receptors redistribute to caveolae in response to receptor occupation. In this study, we tested the hypothesis that adenosine A_1 receptors would translocate to caveolae in the presence of agonists. Surprisingly, in unstimulated rat cardiac ventricular myocytes, 67±5% of adenosine A_1 receptors were isolated with caveolae. However, incubation with the adenosine A_1 receptor agonist 2-chlorocyclopentyladenosine induced the rapid translocation of the A_1 receptors from caveolae into non-caveolae plasma membrane, an effect that was blocked by the adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3dipropylxanthine. An adenosine A_1 receptor agonist did not alter the localization of A_1 receptors to caveolae. These data suggest that the translocation of A_1 receptors out of caveolae and away from compartmentalized signaling molecules may explain why activation of ventricular myocyte A_1 receptors are associated with few direct effects.

3.109 High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae

Uittenbogaard, A., Shaul, P.W., Yuhanna, I.S., Blair, A. and Smart, E.J.
J. Biol. Chem., ***275(15)***, *11278-11283 (2000)*

Oxidized LDL (oxLDL) depletes caveolae of cholesterol, resulting in the displacement of endothelial nitric-oxide synthase (eNOS) from caveolae and impaired eNOS activation. In the present study, we determined if the class B scavenger receptors, CD36 and SR-BI, are involved in regulating nitric-oxide synthase localization and function. We demonstrate that CD36 and SR-BI are expressed in endothelial cells, co-fractionate with caveolae, and co-immuno-precipitate with caveolin-1. Co-incubation of cells with 10 *ug/ml* high density lipoprotein (HDL) prevented oxLDL induced translocation of eNOS from caveolae and restored acetylcholine-induced nitric-oxide synthase stimulation. Acetylcholine caused eNOS activation in cells incubated with 10 *ug/ml* oxLDL (10-15 thiobarbituric acid-reactive substances) and blocking antibodies to CD36, whereas cells treated with only oxLDL were unresponsive. Furthermore, CD36-blocking antibodies prevented oxLDL induced redistribution of eNOS. SR-BI-blocking antibodies were used to demonstrate that the effects of HDL are mediate by SR-BI. HDL binding to SR-BI maintained the concentration of caveola-associated cholesterol by promoting the uptake of cholesterol esters, thereby preventing oxLDL induced depletion of caveola cholesterol We conclude that CD36 mediates the effects of oxLDL on caveola composition and eNOS activation. Furthermore, HDL prevents oxLDL from decreasing the capacity for eNOS activation by preserving the cholesterol concentration in caveolae and, thereby maintaining the subcellular location of eNOS.

3.110 Gastropod mollusc aliphatic alcohol oxidase: subcellular localisation and properties

Grewal, N., Parveen, Z., Large, A., Perry, C. and Connock, M.
Comp. Biochem. Biophys., ***125****, 543-554 (2000)*

The digestive gland and other tissues of several species of terrestrial gastropod mollusc contain an aliphatic alcohol oxidase activity (EC1.1.3.13). The enzyme is FAD dependent, consumes oxygen and generates hydrogen peroxide and the corresponding aldehyde. Saturated primary alcohols arc favoured as substrates with octanol preferred with an apparent K_m of 3-4 μM**.** The activity is clearly distinguishable from previously reported molluscan aromatic alcohol oxidase (EC1.1.3.7) on the basis of FAD dependence, sensitivity to heat treatment and hi-h salt concentration and with regard to substrate preferences. The aliphatic alcohol oxidase is membrane associated and most likely localised to the endoplasmic reticulum. Extraction of membranes with 1% Igipal solubilises the enzyme in active form. This enzyme is a further example of an oxidase apparently restricted to molluscs.

3.111 *CCC1* suppresses mitochondrial damage in the yeast model of Friedreich's ataxia by limiting mitochondrial iron accumulation

Chen, O.S. and Kaplan J.
*J. Biol. Chem****., 275(11)****, 7626-7632 (2000)*

Deletion of *YFH1* in *Saccharomyces cerevisiae* leads to a loss of respiratory competence due to excessive mitochondrial iron accumulation. A suppressor screen identified a gene, *CCC1*, that maintained respiratory function in a Δ*yfh1* yeast strain regardless of extracellular iron concentration. *CCC1* expression prevented excessive mitochondrial iron accumulation by limiting mitochondrial iron uptake rather than by increasing mitochondrial iron egress. Expression of *CCC1* did not result in sequestration of iron in membranous compartments or cellular iron export. *CCC1* expression in wild type cells resulted in increased expression of the high affinity iron transport system composed of *FET3* and *FTR1*, suggesting that intracellular irons is not sensed by the iron-dependent transcription factor Aft1p. Introduction of $AFT1^{up}$, a constitutive allele of the iron transcription factor, *AFT1,* that also leads to increased high affinity iron transport did not prevent Δ*yfh1* cells from becoming respiratory-incompetent. Although the mechanism by which *CCC1* expression affects cytosolic iron is not known, the data suggest that excessive mitochondrial iron accumulation only occurs when cytosolic free iron levels are high.

3.112 Subcellular localization of presenilins: association with a unique membrane pool in cultured cells

Kim, S.H., Lah, J.J., Thinakaran, G., Levey, A. and Sisodia S.S
Neurobiol. Dis., ***7(2)****, 99-117 (2000)*

We have investigated the subcellular distribution of presinilin-1 (PS1) and presenilin-2 (PS2) in a variety of mammalian cell lines. In **Iodixanol** –based density gradients, PS1 derivatives show a biphasic distribution, cofractionating with membranes containing ER-resident proteins and an additional population of membranes with low buoyant density that do not contain markers of the Golgi complex, ERGIC, COP II vesicles, ER exit compartment, COP II receptor, SNARE, trans-Golgi network, caveolar membranes, or endocytic vesicles. Confocal immunofluorescence and immunoelectron microscopy studies fully supported the fractionation studies. These data suggest that PS1 fragments accumulate in a unique subcompartment(s) of the ER or ER to Golgi trafficking intermediates. Interestingly, the FAD-linked PS1 variants show a marked redistribution toward the heavier region of the gradient. Finally, and in contrast PS1 and PS2 fragments are detected preponderantly in more densely sedimenting membranes, suggesting that the subcellular compartments in which these molecules accumulate are distinct.

3.113 Mouse hepatitis virus replicase proteins associate with two distinct populations of intracellular membranes

Sims, A.C., Ostermann, J. and Denison, M.R.
J. Virol., ***74(12)****, 5647-5654 (2000)*

The coronavirus replicase gene (gene1) is translated into two co-amino-terminal polyproteins that are proteolytically processed to yield more than 15 mature proteins. Several gene1 proteins have been shown to localize at sites of viral RNA synthesis in the infected cell cytoplasm, notably on late endosomes at early times of infection. However, both immunofluorescence and electron microscopy studies have also detected gene 1 proteins at sites distinct from the putative sites of viral RNA synthesis or virus assembly. In this study, mouse hepatitis virus (MHV)-infected cells were fractionated and analyzed to determine if gene 1 proteins segregated to more than one membrane population. Following differential centrifugation of lysates

of MHV-infected DBT cells, gene 1 proteins as well as the structural N and M proteins were detected almost exclusively in a high-speed small membrane pellet. Following fractionation of the small membrane pellet on an **iodixanol** density gradient, the gene 1 proteins p28 and helicase cofractionated with dense membranes (1.12 to 1.13 g/ml) that also contained peak concentrations of N. In contrast, p65 and p1a-22 were detected in a distinct population of less dense membranes (1.05 to 1.09 g/ml). Viral RNA was detected in membrane fractions containing helicase, p28, and N but not fractions containing p65 and p1a-22. LAMP-1, a marker for late endosomes and lysosomes, was detected in both membrane populations. These results demonstrate that multiple gene 1 proteins segregate into two biochemically distinct but tightly associated membrane populations and that only one of the populations appears to be a site for viral RNA synthesis. The results further suggest that p28 is a component of the viral replication complex whereas the gene 1 proteins p1a-22 and p65 may serve roles during infection that are distinct from viral RNA transcription or replication.

3.114 Characterization of isolated acidocalcisomes of Trypanosoma cruzi
Scott, D.A. and Docampo, R.
J. Biol. Chem., ***275(31)***, *24215-24221 (2000)*

The acidocalcisome is an acidic calcium store in trypanosomatids, with a vacuolar-type proton-translocating pyrophosphatase (V-H^+-PPase) located in the membrane. In this paper, we describe a new method using **iodixanol** density gradients for purification of the acidocalcisomes from *Trypanosoma cruzi* epimastigotes. Pyrophosphatase assays indicated that the isolated organelle was at least 60-fold purified compared with the large organelle (10,000xg) fraction. Assays for other organelles generally indicated no enrichment in the acidocalcisome fraction; glycosomes were concentrated 5-fold. Vanadate-sensitive ATP-driven Ca^{2+} uptake (Ca^{2+}-ATPase) activity was detectable in isolated acidocalcisome, but ionophore experiments indicated that it was not acidic. However, when pyrophosphate was added, the organelle acidified and rate of Ca^{2+} uptake increased. Use of the indicator Oxonol VI showed that PPase activity generated a membrane potential. Use of sulfate or nitrate in place of chloride in the assay buffer did not affect V-H^+-ATPase activity, but there was less activity with gluconate. Organelle acidification was countered by the chloride/proton symport cycloprogidiosin. No vacuolar H^+-ATPase activity was detectable in isolated acidocalcisomes. However, immunoblots showed the presence of at least a membrane-bound V-H^+-ATPase subunit, while experiments employing permeabilized epimastigotes suggested that vacuolar H^+-ATPase and V-H^+-PPase activities are present in the same Ca^{2+}-containing compartment.

3.115 The existence of a lysosomal redox chain and the role of ubiquinone
Gille, L. and Nohl, H.
Arch. Biochem. Biophys., ***375(2)***, *347-354 (2000)*

Several studies concerning the distribution of ubiquinone (UQ) in the cell report a preferential accumulation of this biogenic quinone in mitochondria, plasma membranes, Golgi vesicles, and lysosomes. Except for mitochondria, no recent comprehensive experimental evidence exists on the particular function of UQ in these subcellular organelles. The aim of a recent study was to elucidate whether UQ is an active part of an electron-transfer system in lysosomes. In the present work, a lysosomal fraction was prepared from a light mitochondrial fraction of rat liver by isopycnic centrifugation. The purity of our preparation was verified by estimation of the respective marker enzymes. Analysis of lysosomes for putative redox carriers and redox processes in lysosomes was carried out by optical spectroscopy, HPLC, oxymetry, and ESR techniques. UQ was detected in an amount of 2.2 nmol/mg of protein in lysosomes. Furthermore, a b -type cytochrome and a flavin-adenine dinucleotide (FAD) were identified as other potential electron carriers. Since NADH was reported to serve as a substrate of UQ redox chains in plasma membranes, we also tested this reductant in lysosomes. Our experiments demonstrate a NADH-dependent reduction of UQ by two subsequent one-electron-transfer steps giving rise to the presence of ubisemiquinone and an increase of the ubiquinol pool in lysosomes. Lysosomal NADH oxidation was accompanied by an approximately equimolar oxygen consumption, suggesting that O_2 acts as a terminal acceptor of this redox chain. DMPO/OH spin adducts were detected by ESR in NADH-supplemented lysosomes, suggesting a univalent reduction of oxygen. The kinetic analysis of redox changes in lysosomes revealed that electron carriers operate in the sequence NADH > FAD > cytochrome b > ubiquinone > oxygen. By using the basic spin label TEMPAMINE, we showed that the NADH-related redox chain in lysosomes supports proton accumulation in lysosomes. In contrast to the hypothesis that UQ in lysosomes is simply a waste product of autophagy in the cell, we demonstrated that this lipophilic electron carrier is a native constituent

of a lysosomal electron transport chain, which promotes proton translocation across the lysosomal membrane.

3.116 Caveolar structure and protein sorting are maintained in NIH cells independent of glycosphingolipid depletion
Shu, L et al
Arch. Biochem. Biophys., ***373(1)****, 83-90 (2000)*

Glycosphingolipids have been proposed to be critical components of cluster lipids within cell membranes that serve as rafts for the attachment and sorting of proteins to the cell membrane. Density gradient centrifugation was used to isolate and to ascertain the lipid composition of caveolin-enriched membranes. These membranes demonstrated a significant enrichment of sphingolipids and cholesterol containing up to 20% and 30%, respectively, of the cellular glucosylceramide and lactosylceramide. A specific inhibitor of glucosylceramide synthase, d-threo-1-phenyl-2-palmitoyl-3-pyrrolidino-propanol, was used to test the hypothesis that glycosphingolipids are required for the sorting of proteins to caveolae. When NIH 3T3 cells were depleted of their glucosylceramide based glycosphingolipid mass, the caveolar structure remained intact as determined by electron microscopy and confocal microscopy. The caveolar proteins caveolin and annexin II sorted normally to caveolae, as determined by immunoblotting and confocal microscopy. When the GPI-linked protein B61 was inducibly expressed in these cells, sorting to caveolar membranes occurred normally, even in the presence of glucosylceramide depletion. These observations suggest that protein sorting to caveolae in fibroblasts occurs independently of glycosphingolipid synthesis.

3.117 Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast
Bagnat, M., Keranen, S., Shevchenko, A., Shevchenko, A. and Simons, K.
Proc. Natl. Acad. Sci.,. USA, ***97(7)****, 3254-3259 (2000)*

Lipid rafts, formed by lateral association of sphingolipids and cholesterol, have been implicated in membrane traffic and cell signaling in mammalian cells. Sphingolipids also have been shown to play a role in protein sorting in yeast. Therefore, we wanted to investigate whether lipid rafts exist in yeast and whether these membrane microdomains have analogous function to their mammalian counterparts. We first developed a protocol for isolating detergent-insoluble glycolipid-enriched complexes (DIGs) from yeast cells. Sequencing of the major protein components of the isolated DIGs by mass spectrometry allowed us to identify, among others, Gas1p, Pma1p, and Nce2p. Using lipid biosynthetic mutants we could demonstrate that conditions that impair the synthesis of sphingolipids and ergosterol also disrupt raft association of Gas1p and Pma1p but not the secretion of acid phosphatase. That endoplasmic reticulum (ER)-to-Golgi transport of Gas1p is blocked in the sphingolipid mutant *lcb1-100* raised the question of whether proteins associate with lipid rafts in the ER or later as shown in mammalian cells. Using the sec 18-1 mutant we found that DIGs are present already in the ER. Taken together, our results suggest that lipid rafts are involved in the biosynthetic delivery of proteins to the yeast plasma membrane.

3.118 Dissecting the role of the Golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface
Heino, S. et al
Proc. Natl. Acad. Sci., USA, ***97(15)****, 8375-8380 (2000)*

In this study, we compared the transport of newly synthesized cholesterol with that of influenza hemagglutinin (HA) from the endoplasmic reticulum to the plasma membrane. The arrival of cholesterol on the cell surface was monitored by cyclodextrin removal, and HA transport was monitored by surface trypsinization and endoglycosidase H digestion. We found that disassembly of the Golgi complex by brefeldin A treatment resulted in partial inhibition of cholesterol transport while completely blocking HA transport. Further, microtubule depolymerization by nocodazole inhibited cholesterol and HA transport to a similar extent. When the partitioning of cholesterol into lipid rafts was analyzed, we found that newly synthesized cholesterol began to associate with low-density detergent-resistant membranes rapidly after synthesis, before it was detectable on the cell surface, and its raft association increased further upon chasing. When cholesterol transport was blocked by using 15°C incubation, the association of newly synthesized cholesterol with low-density detergent-insoluble membranes was decreased and cholesterol accumulated in a fraction with intermediate density. Our results provide evidence for the partial contribution of the Golgi complex to the transport of newly synthesized cholesterol to the cell surface and suggest that detergent-resistant membranes are involved in the process.

3.119 Palmitoylation of caveolin-1 is required for cholesterol binding, chaperone complex formation, and rapid transport of cholesterol to caveolae
Uittenbogaard, A. and Smart, E.J.
J. Biol. Chem., ***275(33)***, *25595-25599 (2000)*

We previously demonstrated that a caveolin-chaperone complex transports newly synthesized cholesterol from the endoplasmic reticulum through the cytoplasm to caveolae. Caveolin-1 has a 33-amino acid hydrophobic domain and three sites of palmitoylation in proximity to the hydrophobic domain. In the present study, we hypothesized that palmitoylation of caveolin-1 is necessary for binding of cholesterol, formation of a caveolin-chaperone transport complex, and rapid direct transport of cholesterol to caveolae. To test this hypothesis, four caveolin-1 constructs were generated that substituted an alanine for a cysteine at position 133, 143, or 156 or all three sites (triple mutant). These mutated caveolins and wild type caveolin-1 were stably expressed in the lymphoid cell line, L1210-JF which does not express caveolin-1, does not form a caveolin-chaperone complex, and does not transport newly synthesized cholesterol to caveolae. All of the caveolins were expressed and the proteins localized to plasma membrane caveolae. Wild type caveolin-1 and mutant 133 assembled into complete transport complexes and rapidly (10-20 min) transported cholesterol to caveolae. Caveolin mutants 143 and 156 did not assemble into complete transport complexes, weakly associated with cholesterol, and transported small amounts of cholesterol to caveolae. The triple mutant did not assemble into complete transport complexes and did not associate with cholesterol. We conclude that palmitoylation of caveolin-1 at positions 143 and 156 is required for cholesterol binding and transport complex formation.

3.120 Mechanism of residence of cytochrome b(5), a tail-anchored protein, in the endoplasmic reticulum
Pedrazzini, E., Villa, A., Longhi, R., Bulbarelli, A. and Borgese, N.
J. Cell Biol., ***148(5)***, *899-913 (2000)*

Endoplasmic reticulum (ER) proteins maintain their residency by static retention, dynamic retrieval, or a combination of the two. Tail-anchored proteins that contain a cytosolic domain associated with the lipid bilayer via a hydrophobic stretch close to the COOH terminus are sorted within the secretary pathway by largely unknown mechanisms. Here, we have investigated the mode of insertion in the bilayer and the intracellular trafficking of cytochrome b(5) (b[5]), taken as a model for ER-resident tail-anchored proteins. We first demonstrated that b(5) can acquire a transmembrane topology posttranslationally, and then used two tagged versions of b(5), N-glyc and O-glyc b(5), containing potential N- and O-glycosylation sites, respectively, at the COOH-terminal lumenal extremity, to discriminate between retention and retrieval mechanisms. Whereas the N-linked oligosaccharide provided no evidence for retrieval from a downstream compartment, a more stringent assay based on carbohydrate acquisition by O-glyc b(5) showed that b(5) gains access to enzymes catalyzing the first steps of O-glycosylation. These results suggest that b(5) slowly recycles between the ER and the *cis*-Golgi complex and that dynamic retrieval as well as retention are involved in sorting of tail-anchored proteins.

3.121 The role of the COOH terminus of Sec2p in the transport of post-Golgi vesicles
Elkind., N.B., Walch-Solimena, C. and Novick, P.J.
J. Cell Biol., ***149(1)***, *95-110 (2000)*

Sec2p is required for the polarized transport of secretory vesicles in *S cerevisiae*. The Sec2p NH_2 terminus encodes an exchange factor for the Rab protein Sec4p. Sec2p associates with vesicles and in Sec2p COOH-terminal mutants Sec4p and vesicles no longer accumulate at bud tips. Thus, the Sec2p COOH terminus functions in targeting vesicles, however, the mechanism of function is unknown. We found comparable exchange activity for truncated and full-length Sec2 proteins, implying that the COOH terminus does not alter the exchange rate. Full-length Sec2-GFP, similar to Sec4p, concentrates at bud tips. A COOH-terminal 58-amino acid domain is necessary but not sufficient for localization. Sec2p localization depends on actin, Myo2p and Seclp, Sec6p, and Sec9p function. Full-length, but not COOH-terminally truncated Sec2 proteins are enriched on membranes. Membrane association of full-length Sec2p is reduced in sec6-4 and sec9-4 backgrounds at 37°C but unaffected at 25°C. Taken together, these data correlate loss of localization of Sec2 proteins with reduced membrane association. In addition, Sec2p membrane attachment is substantially Sec4p independent, supporting the notion that Sec2p interacts with membranes via an unidentified Sec2p receptor, which would increase the accessibility of Sec2p exchange activity for Sec4p.

3.122 Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. Evidence for two pathways of iron uptake
Yun, C-W. et al
J. Biol. Chem., ***275(14)***, *10709-10715 (2000)*

In the yeast *Saccharomyces cerevisiae*, uptake of iron is largely regulated by the transcription factor Aftl. cDNA microarrays were used to identify new iron and *AFT1*-regulated genes. Four homologous genes regulated as part of the *AFT1*-regulon (*ARN1-4*) were predicted to encode members of a subfamily of the major facilitator superfamily of transporters. These genes were predicted to encode proteins with 14 membrane spanning domains and were from 26 to 53% identical at the amino acid level. *ARN3* is identical to SIT1, which is reported to encode a ferrioxamine B permease. Deletion of *ARN3* did not prevent yeast from using ferrioxamine B as an iron source; however, deletion of *ARN3* and *FET3*, a component of the high affinity ferrous iron transport system, did prevent uptake of ferrioxamine bound iron and growth on ferrioxamine as an iron source. The siderophore-mediated transport system and the high affinity ferrous iron transport system were localized to separate cellular compartments. Epitope-tagged Arn3p was expressed in intracellular vesicles that co-sediment with the endosomal protein Pepl2. In contrast, Fet3p was expressed on the plasma membrane and was digested by extracellular proteases. These data indicate that S. cerevisiae has two pathways for ferrrioxamine-mediated iron uptake, one occurring at the plasma membrane and the other occurring in an intracellular compartment**.**

3.123 Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains
Lockwich, T.P. et al
J. Biol. Chem., ***275(16)***, *11934-11942 (2000)*

Trpl has been proposed as a component of the store-operated Ca^{2+} entry (SOC) channel. However, neither the molecular mechanism of SOC nor the role of Trp in this process is yet understood. We have examined possible molecular interactions involved in the regulation of SOC and Trpl and report here for the first time that Trpl is assembled in signaling complex associated with caveolin-scaffolding lipid raft domains. Endogenous hTrpl and caveolin-1 were present in low density fractions of Triton X-100-extracted human submandibular gland cell membranes. Depletion of plasma membrane cholesterol increased Triton X-100 solubility of Trpl and inhibited carbachol-stimulated Ca^{2+} signaling. Importantly, thapsigargin stimulated Ca^{2+} influx, but not internal Ca^{2+} release, and inositol 1,4,5-triphosphate (IP_3) stimulated I_{soc} were also attenuated. Furthermore, both anti-Trp1 and anti-caveolin-1 antibodies co-immunoprecipitated hTrpl, caveolin-1, $G\alpha_{q/11}$ and IP_3 receptor-type 3 (IP_3R3). These results demonstrate that caveolar microdomains provide a scaffold for (i) assembly of key Ca^{2+} signaling proteins into a complex and (ii) coordination of the molecular interactions leading to the activation of SOC. Importantly, we have shown that Trpl is also localized in this microdomain where it interacts with one or more components of this complex, including IP_3 R3. This finding is potentially important in elucidating the physiological function of Trp.

3.124 Enterotoxigenic *Escherichia coli* secretes active heat-labile enteroxin via outer membrane vesicles
Horstman, A.L. and Kuehn, M.J.
J. Biol. Chem., ***275(17)***, *12489-12496 (2000)*

Escherichia coli and other Gram-negative bacteria produce outer membrane vesicles during normal growth. Vesicles may contribute to bacterial pathogenicity by serving as vehicles for toxins to encounter host cells. Enterotoxigenic *E.coli* (ETEC) vesicles were isolated from culture supernatants and purified on velocity gradients, thereby removing any soluble proteins and contaminants from the crude preparation. Vesicle protein profiles were similar but not identical to outer membranes and differed between strains. Most vesicle proteins were resistant to dissociation, suggesting they were integral or internal. Thin layer chromatography revealed that major outer membrane lipid components are present in vesicles. Cytoplasmic membranes and cytosol were absent in vesicles; however, alkaline phosphatase and AcrA, periplasmic residents, were localized to vesicles. In addition, physiologically active heat-labile enterotoxin (LT) was associated with ETEC vesicles. LT activity correlated directly with the gradient peak of vesicles, suggesting specific association, but could be removed from vesicles under dissociating conditions. Further analysis revealed that LT is enriched in vesicles and is located both inside and on the exterior of vesicles. The distinct protein composition of ETEC vesicles and their ability to carry toxin may contribute to the pathogenicity of ETEC strains.

3.125 Biosynthesis of a major lipofuscin fluorophore in mice and humans with *ABCR*–mediated retinal and macular degeneration

Mata, N.L., Weng, J. and Travis, G.H.
*Proc. Natl. Acad. Sci. USA, **97(13)**, 7154-7159 (2000)*

Increased accumulation of lipofuscin in cells of the retinal pigment epithelium (RPE) is seen in several forms of macular degeneration, a common cause of blindness in humans. A major fluorophore of lipofuscin is the toxic *bis*-retinoid, *N*-retinylidene-*N*-retinylethanolamine (A2E). Previously, we generated mice with a knockout mutation in the *abcr* gene. This gene encodes rim protein (RmP), an ATP-binding cassette transporter in rod outer segments. Mice lacking RmP accumulate A2E in RPE cells at a greatly increased rate over controls. Here, we identify three precursors of A2E in ocular tissues from *abcr*/mice and humans with *ABCR*-mediated recessive macular degenerations. Our results corroborate the scheme proposed by C. A. Parish, M. Hashimoto, K. Nakanishi, J. Dillon & J. Sparrow *[Proc. Natl. Acad. Sci. USA* (1998) 95,14609-14613], for the biosynthesis of A2E: (i) condensation of all-*trans*-retinaldehyde *(*all-*trans*-RAL*)* with phosphatidylethanolamine to form a Schiff base; (ii) condensation of the amine product with a second all-*trans*-RAL to form a *bis*-retinoid; (iii) oxidation to yield a pyridinium salt; and (iv) hydrolysis of the phosphate ester to yield A2E. The latter two reactions probably occur within RPE phagolysosomes. As predicted by this model, formation of A2E was completely inhibited when *abcr* / mice were raised in total darkness. Also, once formed, A2E was not eliminated by the RPE. These data suggest that humans with retinal or macular degeneration caused by loss of RmP function may slow progression of their disease by limiting exposure to light. The precursors of A2E identified in this study may represent pharmacological targets for the treatment of *ABCR*-mediated macular degeneration.

3.126 Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIIb

Plant PJ. et al
*J. Cell Biol., **149(7)**, 1473-1483 (2000).*

Nedd4 is a ubiquitin protein ligase (E3) containing a C2 domain, three or four WW domains, and a ubiquitin ligase HECT domain. We have shown previously that the C2 domain of Nedd4 is responsible for its Ca2+-dependent targeting to the plasma membrane, particularly the apical region of epithelial MDCK cells. To investigate this apical preference, we searched for Nedd4-C2 domain-interacting proteins that might be involved in targeting Nedd4 to the apical surface. Using immobilized Nedd4-C2 domain to trap interacting proteins from MDCK cell lysate, we isolated, in the presence of Ca2+, a ~35-40-kD protein that we identified as annexin XIII using mass spectrometry. Annexin XIII has two known isoforms, a and b, that are apically localized, although XIIIa is also found in the basolateral compartment. In vitro binding and coprecipitation experiments showed that the Nedd4-C2 domain interacts with both annexin XIIIa and b in the presence of Ca2+, and the interaction is direct and optimal at 1 μM Ca2+. Immunofluorescence and immunogold electron microscopy revealed colocalization of Nedd4 and annexin XIIIb in apical carriers and at the apical plasma membrane. Moreover, we show that Nedd4 associates with raft lipid microdomains in a Ca2+ -dependent manner, as determined by detergent extraction and floatation assays. These results suggest that the apical membrane localization of Nedd4 is mediated by an association of its C2 domain with the apically targeted annexin XIIIb.

3.127 Mutation of conserved aspartates affects maturation of both aspartate mutant and endogenous presenilin 1 and presenilin 2 complexes
Yu, G. et al
J. Biol. Chem., ***275(35)***, *27348-27353 (2000)*

Presenilin (PS1 and PS2) holoproteins are transiently incorporated into low molecular weight (MW complexes. During subsequent incorporation into a higher MW complex, they undergo endoproteolysis to generate stable N- and C-terminal fragments. Mutation of either of two conserved aspartate residues in transmembrane domains inhibits both presenilin-endoproteolysis and the proteolytic processing of β-amyloid precursor protein and Notch. We show that although PS1/PS2 endoproteolysis is not required for inclusion into the higher MW N- and C-terminal fragment-containing complex, aspartate mutant holoprotein presenilins are not incorporated into the high MW complexes. Aspartate mutant presenilin holoproteins also preclude entry of endogenous wild type PS1/PS2 into the high MW complexes but do not affect the incorporation of wild type holoproteins into lower MW holoprotein complexes. These data suggest that the loss of function effects of the aspartate mutants result in altered PS complex maturation and argue that the functional presenilin moieties are contained in the high molecular weight complexes.

3.128 ^{31}P NMR spectroscopy of *Trypanosoma brucei, Trypanosoma cruzi*, and *Leishmania major*
Moreno, B. et al
J. Biol. Chem., ***275(37)***, *28356-28362 (2000)*

High resolution ^{31}P nuclear magnetic resonance spectra at 303.6 MHz (corresponding to a ^{1}H resonance frequency of 750 MHz) have been obtained of perchloric acid extracts of *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania major*, the causative agents of African sleeping sickness, Chagas'disease, and leishmaniasis. Essentially complete assignments have been made based on chemical shifts and by direct addition of authentic reference compounds. The results indicate the presence of high levels of short chain condensed polyphosphates: di-, tri-, tetra-, and pentapolyphosphate. ^{31}P NMR spectra of purified *T. brucei, T. cruzi* and *L. major* acidocalcisomes, calcium and phosphorus storage organelles, indicate that polyphosphates are abundant in these organelles and have an average chain length of 3.11-3.39 phosphates. In the context of the recent discovery of several pyrophosphate-utilizing enzymes in trypanosomatids, the presence of these inorganic polyphosphates implies a critical role for these molecules in these parasites and a potential new route to chemotherapy.

3.129 Epidermal growth factor-mediated caveolin recruitment to early endosomes and MAPK activation
Pol, A., Lu, A., Pons, M., Peiro, S. and Enrich, C.
J. Biol. Chem., ***275(39)***, *30566-30572 (2000)*

The endocytic compartment of eukaryotic cells is a complex intracellular structure involved in sorting, processing, and degradation of a great variety of internalized molecules. Recently, the uptake through caveolae has emerged as an alternative internalization pathway, which seems to be directly related with some signal transduction pathways. However, the mechanisms, molecules, and structures regulating the transport of caveolin from the cell surface into the endocytic compartment are largely unknown. In this study, normal quiescent fibroblasts (normal rat kidney (NRK)) were used to demonstrate that epidermal growth factor causes partial redistribution of caveolin from the cell surface into a cellubrevin early endocytic compartment. Treatment of NRK cells with cytochalasin D or latrunculin A inhibit this pathway and the concomitant activation of Mek and mitotic-activated protein (MAP) kinase; however, if cells were pre-treated with filipin, cytochalasin D does not inhibit the phosphorylation of MAP kinase induced by epidermal growth factor. From these results we conclude that in NRK cells the intact actin cytoskeleton is necessary for the EGF-mediated transport of caveolin from the cell surface into the early endocytic compartment and the activation of MAP kinase pathway.

3.130 Dystrophin associates with a caveolae of rat cardiac myocytes

Doyle, D.D. et al

*Circulation Res., **87**, 480-488 (2000)*

The possibility of an interaction between the cytoskeletal protein dystrophin and cell surface caveolae in the mammalian myocardium was investigated by several techniques. Caveolin (cav)-3-enriched, detergent-insoluble membranes isolated from purified ventricular sarcolemma by density-gradient fractionation were found to contain dystrophin and dystroglycan. Further purification of cav-3-containing membranes by immunoprecipitation using anti-cav-3-coated magnetic beads yielded dystrophin but not always dystroglycan. Electron microscopic analysis of precipitated material revealed caveola-sized vesicular profiles that could be double-labeled with anti-dystrophin and anti-cav-3 antibodies. In contrast, immunoprecipitation of membranes with anti-dystrophin-coated beads yielded both cav-3 and dystroglycan. Electron microscopic analysis of this material showed heterogeneous membrane profiles, some of which could be decorated with anti-cav-3 antibodies. To confirm that dystrophin and cav-3 were closely associated in cardiac myocytes, we verified that dystrophin was also present in immunoprecipitated cav-3-containing membranes from detergent extracts, as well as in sonicated extracts of purified ventricular myocytes. Confocal immunofluorescence microscopy of ventricular and atrial cardiac myocytes showed that the cellular distributions of cav-3 and dystrophin partially overlapped. Immuno-electron micrographs of thin sections of rat atrial myocytes revealed a fraction of dystrophin molecules that are in apparently close apposition to caveolae. These results suggest that a subpopulation of dystrophin molecules interacts with cardiac myocyte caveolae in vivo and that some of the dystrophin is engaged in linking cav-3 with the dystroglycan complex.

3.131 Chemical stimulation of synaptosomes modulates α-Ca^{2+}/calmodulin-dependent protein kinase II mRNA association to polysomes

Bagni, C., Mannucci, L., Dotti, C.G. and Amaldi, F.

*J. Neurosci., **20 RC76**, 1-6 (2000)*

The presence of specific mRNAs in dendrites and at synapses is well established, but a direct and reliable demonstration that they are associated with polysomes is still missing. To address this point we analyzed the polysomal association of the mRNAs for the α-subunit of Ca^{2+} /calmodulin-dependent protein kinase II (α-CaMKII), for type 1 inositol 1,4,5-trisphosphate receptor (InsP3Rl) and for the activity-regulated cytoskeleton-associated protein (Arc) in a synaptosomal preparation devoid of contaminating material from neuronal and glial perikarya. We show that a fraction of α-CaMKII, InsP3Rl, and Arc mRNAs present in synaptosomes is indeed associated with polysomes. Moreover, we show that polysomal association of α-CaMKII mRNA, but not InsP3Rl and Arc mRNAs, increases with depolarization of the synaptosomal membrane. Finally, we show that the synthesis of α-CaMKII protein increases with stimulation. Dendritic mRNA recruitment onto polysomes in response to synaptic stimulation might represent one of the mechanisms underlying the processes of learning and memory.

3.132 Mutant presenilin 1 increases the levels of Alzheimer amyloid β-peptide Aβ42 in late compartments of the constitutive secretory pathway
Petanceska, S., Seeger, M., Checler, F. and Gandy, S.
*J. Neurochem., **74**, 1878-1884 (2000)*

Mutations in the presenilin 1 (PS1) gene are associated with autosomal dominant, early-onset, familial Alzheimer's disease and result in increased release of the hyperaggregatable 42-amino acid form of the amyloid β-peptide (Aβ42). To determine which subcellular compartments are potential source(s) of released Aβ42, we compared the levels and spatial segregation of intracellular Aβ40 and Aβ42 peptides between N2a neuroblastoma cells doubly transfected with the "Swedish" familial Alzheimer's disease-linked amyloid precursor protein variant and either wild-type PS1 ($PS1^{wt}$) or familial Alzheimer's disease-linked Δ9 mutant PS1 ($PS1^{\Delta 9}$). As expected, $PSl^{\Delta 9}$-expressing cells had dramatically higher levels of intracellular Aβ42 than did cells expressing $PS1^{wt}$. However, the highest levels of Aβ42 colocalized not with endoplasmic reticulum or Golgi markers but with rab8, a marker for trans-Golgi network (TGN)-to-plasma membrane (PM) transport vesicles. We show that PS1 mutants are capable of causing accumulation of Aβ42 in late compartments of the secretary pathway, generating there a readily releasable source of Aβ42. Our findings indicate that PS1 "bioactivity" localizes to the vicinity of the TGN and/or PM and reconcile the apparent discrepancy between the preponderant concentration of PS1 protein in proximal compartments of the secretary pathway and the recent findings that PS1 "bioactivity" can control γ-secretase-like processing of another transmembrane substrate, Notch, at or near the PM.

3.133 Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland
Nemeth, B.A., Tsang, S.W.Y., Geske, R.S. and Haney, P.
*Pediatr Res., **47**, 444-450 (2000)*

Lactose, the major carbohydrate of human milk, is synthesized in the Golgi from glucose and UDP-galactose. The lactating mammary gland is unique in its requirement for the transport of glucose into Golgi. Glucose transporter-1 (GLUT1) is the only isoform of the glucose transporter family expressed in mammary gland. In most cells, GLUT1 is localized to the plasma membrane and is responsible for basal glucose uptake; in no other cell type is GLUT1 a Golgi resident. To test the hypothesis that GLUT1 is targeted to Golgi during lactation, the amount and subcellular distribution of GLUT1 were examined in mouse mammary gland at different developmental stages. Methods including immunohistochemistry, immunofluorescence, subcellular fractionation, density gradient centrifugation, and Western blotting yielded consistent results. In virgins, GLUT1 expression was limited to plasma membrane of epithelial cells. In late pregnant mice, GLUT1 expression was increased with targeting primarily to basolateral plasma membrane but also with some intracellular signal. During lactation, GLUT1 expression was further increased, and targeting to Golgi, demonstrated by colocalization with the 110-kD coatomer-associated protein β-COP, predominated. Removal of pups 18 d after delivery resulted in retargeting of GLUT1 from Golgi to plasma membrane and a decline in total cellular GLUT1 within 3 h. In mice undergoing natural weaning, GLUT1 expression declined. Changes in the amount and targeting of GLUT1 during mammary gland development are consistent with a key role for GLUT1 in supplying substrate for lactose synthesis and milk production.

3.134 Membrane raft microdomains mediate lateral assemblies for HIV-1 infection
Manes, S. et al
*EMBO reports, **1(2),** 190-196 (2000)*

HIV-1 infection triggers lateral membrane diffusion following interaction of the viral envelope with cell surface receptors. We show that these membrane changes are necessary for infection, as initial gpl2O-CD4 engagement leads to redistribution and clustering of membrane microdomains, enabling subsequent interaction of this complex with HIV-1 co-receptors. Disruption of cell membrane rafts by cholesterol depletion before viral exposure inhibits entry by both X4 and R5 strains of HIV-1, although viral replication in infected cells is unaffected by this treatment. This inhibitory effect is fully reversed by cholesterol replenishment of the cell membrane. These results indicate a general mechanism for HIV-1 envelope glycoprotein-mediated fusion by reorganization of membrane microdomains in the target cell, and offer new strategies for preventing HIV-1 infection.

3.135 Subcellular organization of bile acid amidation in human liver: a key in regulating the biosynthesis of bile salts

Solaas, K., Ulvestad, A., Soreide, O. and Kase, B.F.
*J. Lipid Res., **41**, 1154-1162 (2000)*

To extend our knowledge of how the synthesis of free bile acids and bile salts is related within the hepatocytes, bile acid-CoA:amino acid N-acyltransferase and bile acid-CoA thioesterase activities were measured in subcellular fractions of human liver homogenates. Some bile acids, both conjugated and unconjugated, have been reported to be natural ligands for the farnesoid X receptor (FXR), an orphan nuclear receptor. The conversion of [^{14}C]choloyl-CoA and [^{14}C]chenodeoxycholoyl-CoA into the corresponding tauro- and glyco-bile acids or the free bile acids was measured after high-pressure liquid radiochromatography. There was an enrichment of the N-acyltransferase in the cytosolic and the peroxisomal fraction. Bile acid-CoA thioesterase activities were enriched in the cytosolic, peroxisomal, and mitochondrial fractions. The highest amidation activities of both choloyl-CoA and chenodeoxycholoyl-CoA were found in the peroxisomal fraction (15-58 nmol/mg protein/min). The K_m, was higher for glycine than taurine both in cytosol and the peroxisomal fractions These results show that the peroxisomal de novo synthesis of bile acids is rate limiting for peroxisomal amidation, and the microsomal bile acid-CoA synthetase is rate limiting for the cytosolic amidation. The peroxisomal location may explain the predominance of glyco-bile acids in human bile. Both a cytosolic and a peroxisomal bile acid-CoA thioesterase may, influence the intracellular levels of free and conjugated bile acids.

3.136 Human bleomycin hydrolase regulates the secretion of amyloid precursor protein

Lefterov, I.M., Koldamova, R.P. and Lazo, J.S.
*FASEB J., **14**, 1837-1847 (2000)*

Human bleomycin hydrolase (hBH) is a neutral cysteine protease genetically associated with increased risk for Alzheimer disease. We show here that ectopic expression of hBH in 293APPwt and CHOAPPsw cells altered the processing of amyloid precursor protein (APP) and increased significantly the release of its proteolytic fragment, β amyloid (Aβ). We also found that hBH interacted and colocalized with APP as determined by subcellular fractionation, *in vitro* binding assay, and confocal immunolocalization. Metabolic labeling and pulse-chase experiments, showed that ectopic hBH expression increased secretion of soluble APPα/β products without changing the half-life of cellular APP. We also observed that this increased Aβ secretion was independent of hBH isoforms. Our findings suggest a regulatory role for hBH in APP processing pathways.

3.137 Identification, purification and characterization of an acetoacetyl-CoA thiolase from rat liver peroxisomes

Antonenkov, V.D., Croes, K., Waelkens, E., Van Veldhoven, P.P. and Mannaerts, G.P.
*Eur. J. Biochem., **267**, 2981-2990 (2000)*

Acetoacetyl-CoA specific thiolases catalyse the cleavage of acetoacetyl-CoA into two molecules of acetyl-CoA and the synthesis (reverse reaction) of acetoacetyl-CoA. The formation of acetoacetyl-CoA is the first step in cholesterol and ketone body synthesis. In this report we describe the identification of a novel acetoacetyl-CoA thiolase and its purification from isolated rat liver peroxisomes by column chromatography. The enzyme, which is a homotetramer with a subunit molecular mass of 42 kDa, could be distinguished from the cytosolic and mitochondrial acetoacetyl-CoA thiolases by its chromatographic behaviour, kinetic characteristics and partial internal amino-acid sequences. The enzyme did not catalyse the cleavage of medium or long chain 3-oxoacyl-CoAs. The enzyme cross-reacted with polyclonal antibodies raised against cytosolic acetoacetyl-CoA thiolase. The latter property was exploited to confirm the peroxisomal localization of the novel thiolase in subcellular fractionation experiments.
The peroxisomal acetoacetyl-CoA thiolase most probably catalyses the first reaction in peroxisomal cholesterol and dolichol synthesis. In addition, its presence in peroxisomes along with the other enzymes of the ketogenic pathway indicates that the ketogenic potential of peroxisomes needs to be re-evaluated.

3.138 Human oxytocin receptors in cholesterol-rich vs. cholesterol-poor microdomains of the plasma membrane

Gimpl, G. and Fahrenholz, F.
*Eur. J. Biochem., **267**, 2483-2497 (2000)*

We analyzed the properties of a G protein-coupled receptor localized in cholesterol-poor vs. cholesterol-rich microdomains of the plasma membrane. For this purpose, the human oxytocin receptor, which is very sensitive against alterations of the membrane cholesterol level, was stably expressed in HEK293 cells, To calculate the total number of receptors independent of ligand binding studies, the oxytocin receptor was tagged with an enhanced green fluorescent protein (EGFP) which did not change the functional properties of the receptor. Only 1% of the oxytocin receptors were present in cholesterol-rich detergent-insoluble domains. In contrast, employing a detergent-free fractionation scheme that preserves the functional activity of the receptor, we detected 10-15% of the receptors in cholesterol-rich low-density membranes and therein the high-affinity state receptors were twofold enriched. In cholesterol-poor vs. cholesterol-rich domains, high-affinity oxytocin receptors behaved similar with respect to their agonist binding kinetics and GTP sensitivity. However, high-affinity oxytocin receptors localized in cholesterol-rich low-density membranes showed a markedly enhanced (t, ≈ threefold) stability at 37°C as compared with the oxytocin receptors localized in the cholesterol-poor high-density membranes. Addition of cholesterol to the high-density membranes fully protected the oxytocin receptors against loss of function. The importance of cholesterol to stabilize the oxytocin receptor was supported in experiments with solubilized receptors. Cholesterol markedly delayed the inactivation of oxytocin receptors solubilized with Chapso. In conclusion, the data of this report suggest that functional properties of heptahelical receptor proteins could differ in dependence of their localization in different membrane microdomains.

3.139 Involvement of gangliosides in GPI-anchored neuronal cell adhesion molecule TAG-1 signaling in lipid rafts

Kasahara, K. et al
*J. Biol. Chem., **275(44)**, 34701-34709 (2000)*

The association of ganglioside GD3 with TAG-1, a glycosylphosphatidylinositol (GPI)-anchored neuronal cell adhesion molecule, was examined by coimmunoprecipitation experiments. Previously, we have shown that the anti-ganglioside GD3 antibody (R24) immunoprecipitated the src-family kinase Lyn from the rat cerebellum, and R24 treatment of primary cerebellar cultures induced Lyn activation and rapid tyrosine phosphorylation of an 80-kDa protein (p80). We now report that R24 coimmunoprecipitates a 135-kDa protein (p135) from primary cerebellar cultures. Treatment with phosphatidylinositol-specific phospholipase C revealed that p135 was GPI-anchored to the membrane. It was identified as TAG-1 by sequential immunoprecipitation with an anti-TAG-1 antibody. Antibody-mediated crosslinking of TAG-1 induced Lyn activation and rapid tyrosine phosphorylation of p80. Selective inhibitor for src-family kinases reduced the tyrosine phosphorylation of p80. Sucrose density gradient analysis revealed that the TAG-1 and tyrosine-phosphorylated p80 in cerebellar cultures were present in the lipid raft fraction. These data show that TAG-1 transduces signals via Lyn to p80 in the lipid rafts of the cerebellum. Furthermore, degradation of cell-surface glycosphingolipids by endoglycoceramidase induced an alteration of TAG-1 distribution on an **OptiPrep** gradient and reduced the TAG-1 mediated Lyn activation and tyrosine phosphorylation of p80. These observations suggest that glycosphingolipids are involved in TAG-1 mediated signaling in lipid rafts.

3.140 Presence of oxidized cholesterol in caveolae uncouples active platelet-derived growth factor receptors from tyrosine kinase substrates

Liu, P., Wang, P-y., Michaely, P., Zhu, M. and Anderson, R.G.W.
*J. Biol. Chem., **275(41)**, 31648-31654 (2000)*

Platelet-derived growth factor receptor β (PDGFRβ) in fibroblasts is concentrated in caveolae where it controls the tyrosine phosphorylation of multiple proteins. Caveolae are enriched in cholesterol and sphingolipids, but the role of these lipids in PDGFR signal transduction is unknown. We report that introduction of cholest-4-en-3-one into caveolae membranes uncouples PDGFR autophosphorylation from tyrosine phosphorylation of neighboring proteins. Cholest-4-en-3-one appears to interfere with the normal interaction between PDGFR and its partners. The results suggest that tightly packed caveolae lipids form a membrane platform that functions as a lipid scaffold for organizing the molecular interactions of multiple signaling pathways.

3.141 Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation and autophagy
Kegel, K.B. et al
J. Neurosci., ***20(19)***, *7268-7278 (2000)*

An expansion of polyglutamines in the N terminus of huntingtin causes Huntington's disease (HD) and results in the accrual of mutant protein in the nucleus and cytoplasm of affected neurons. How mutant huntingtin causes neurons to die is unclear, but some recent observations suggest that an autophagic process may occur. We showed previously that huntingtin markedly accumulates in endosomal-lysosomal organelles of affected HD neurons and, when exogenously expressed in clonal striatal neurons, huntingtin appears in cytoplasmic vacuoles causing cells to shrink. Here we show that the huntingtin-enriched cytoplasmic vacuoles formed *in vitro* internalized the lysosomal enzyme cathepsin D in proportion to the polyglutamine-length in huntingtin. Huntingtin-labeled vacuoles displayed the ultrastructural features of early and late autophagosomes (autolysosomes), had little or no overlap with ubiquitin, proteasome, and heat shock protein 70/heat shock cognate 70 immunoreactivities, and altered the arrangement of Golgi membranes mitochondria, and nuclear membranes. Neurons with excess cytoplasmic huntingtin also exhibited increased tubulation of endosomal membranes. Exogenously expressed human full-length wild-type and mutant huntingtin codistributed with endogenous mouse huntingtin in soluble and membrane fractions, whereas human N-terminal huntingtin products were found only in membrane fractions that contained lysosomal organelles. We speculate that mutant huntingtin accumulation in HD activates the endosomal-lysosomal system, which contributes to huntingtin proteolysis and to an autophagic process of cell death.

3.142 The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress
Greeve, I. et al
J. Neurosci., ***20(19)***, *7345-7352 (2000)*

In Alzheimer's disease (AD) brains, selected populations of neurons degenerate heavily, whereas others are frequently spared from degeneration. To address the cellular basis for this selective vulnerability of neurons in distinct brain regions, we compared gene expression between the severely affected inferior temporal lobes and the mostly unaffected fronto-parietal cortices by using an mRNA differential display. We identified *seladin-1*, a novel gene, which was down regulated in large pyramidal neurons in vulnerable regions in AD but not control brains. Seladin- 1 is a human homolog of the *DIMINUTO/DWARF1* gene described in plants and *Caenorhabditis elegans*. Its sequence shares similarities with flavin-adenin-dinucleotide (FAD)-dependent oxidoreductases. In human control brain, *seladin-1* was highly expressed in almost all neurons. In PC12 cell clones that were selected for resistance against AD-associated amyloid-β peptide (Aβ)-induced toxicity, both mRNA and protein levels of seladin-1 were approximately threefold higher as compared with the non-resistant wild-type cells. Functional expression of seladin-1 in human neuroglioma H4 cells resulted in the inhibition of caspase 3 activation after either Aβ-mediated toxicity or oxidative stress and protected the cells from apoptotic cell death. In apoptotic cells, however, endogenous seladin-1 was cleaved to a 40 kDa derivative in a caspase-dependent manner. These results establish that seladin-1 is an important factor for the protection of cells against Aβ toxicity and oxidative stress, and they suggest that seladin-1 may be involved in the regulation of cell survival and death, Decreased expression of seladin-1 in specific neurons may be a cause for selective vulnerability in AD.

3.143 Carboxyl-terminal fragments of Alzheimer β-amyloid precursor protein accumulate in restricted and unpredicted intracellular compartments in presenilin 1 deficient cells
Chen, F. et al
J. Biol. Chem., ***275***, *36794-36802 (2000)*

Absence of functional presenilin 1 (PS1) protein leads to loss of γ-secretase cleavage of the amyloid precursor protein (βAPP), resulting in a dramatic reduction in amyloid β peptide (Aβ) production and accumulation of α- or β-secretase-cleaved C-terminal fragments of βAPP (α- or β-CTFs). The major C-terminal fragment (CTF) in brain was identified as βAPP-CTF-(11-98), which is consistent with the observation that cultured neurons generate primarily Aβ(11-40). In $PS1^{-/-}$ murine neurons and fibroblasts expressing the loss-of-function $PS1_{D385A}$ mutant, CTFs accumulated in the endoplasmic reticulum, Golgi, and lysosomes, but not late endosomes. There were some subtle differences in the subcellular distribution of CTFs in $PS1^{-/-}$ neurons as compared to $PS1_{D385A}$ mutant fibroblasts. However, there was no obvious redistribution of full-length βAPP or of markers of other organelles in either mutant. Blockade of endoplasmic reticulum-to-Golgi trafficking indicated that in $PS1^{-/-}$ neurons (as in normal cells) trafficking of βAPP to the Golgi compartment is necessary before α-and β-secretase cleavage occurs. Thus, while we cannot exclude a specific role for PS1 in trafficking of CTFs, these data argue against a major role in general protein trafficking. These results are more compatible with a role for PS1 either as the actual γ-secretase catalytic activity or in other functions indirectly related to γ-secretase catalysis (e.g. an activator of γ-secretase, a substrate-adaptor for γ-secretase, or delivery of γ-secretase to βAPP-containing compartments.

3.144 Differential targeting of β-adrenergic receptor subtypes and adenyl cyclase to cardiomyocyte caveolae: A mechanism to functionally regulate the cAMP signaling pathway
Rybin, V.O., Xu, X., Lisanti, M.P. and Steinberg, S.F.
J. Biol. Chem., ***275(52)***, *41447-41457(2000)*

Differential modes for β_1 and β_2-adrenergic receptor (AR) regulation of adenylyl cyclase in cardiomyocytes is most consistent with spatial regulation in microdomains of the plasma membrane. This study examines whether caveolae represent specialized subdomains that concentrate and organize these moieties in cardiomyocytes. Caveolae from quiescent rat ventricular cardiomyocytes are highly enriched in β_2-ARs, $G\alpha_i$, protein kinase A, RIIα subunits, caveolin-3, and flotillins (caveolin functional homologues); β_1-ARs, m_2-muscarinic cholinergic receptors, $G\alpha_s$ and cardiac types V/VI adenylyl cyclase distribute between caveolae and other cell fractions, whereas protein kinase A RIα subunits, G protein-coupled receptor kinase-2, and clathrin are largely excluded from caveolae. Cell surface β_2-ARs localize to caveolae in cardiomyocytes and cardiac fibroblasts (with markedly different β_2-AR expression levels), indicating that the fidelity of β_2-AR targeting to caveolae is maintained over a physiologic range of β_2-AR expression. In cardiomyocytes, agonist stimulation leads to a marked decline in the abundance of β_2-ARs (but not β_1-ARs) in caveolae. Other studies show co-immunoprecipitation of cardiomyocytes adenylyl cyclase V/VI and caveolin-3, suggesting their *in vivo* association. However, caveolin is not required for adenylyl cyclase targeting to low density membranes, since adenylyl cyclase targets to low buoyant density membrane fractions of HEK cells that lack prototypical caveolins. Nevertheless, cholesterol depletion with cyclodextrin augments agonist-stimulated cAMP accumulation, indicating that caveolae function as negative regulators of cAMP accumulation. The inhibitory interaction between caveolae and the cAMP signaling pathway as well as domain-specific differences in the stoichiometry of individual elements in the β-AR signaling cascade represent important modifiers of cAMP-dependent signaling in the heart.

3.145 Selective accumulation of raft-associated membrane protein LAT in T cell receptor signaling assemblies

Harder, T. and Kuhn, M.
*J. Cell Biol., **151(2)**, 199-207 (2000)*

Activation of T cell antigen receptor (TCR) induces tyrosine phosphorylations that mediate the assembly of signaling protein complexes. Moreover, cholesterol-sphingolipid raft membrane domains have been implicated to play a role in TCR signal transduction. Here, we studied the assembly of TCR with signal transduction proteins and raft markers in plasma membrane subdomains of Jurkat T leukemic cells. We employed a novel method to immunoisolate plasma membrane subfragments that were highly concentrated in activated TCR-CD3 complexes and associated signaling proteins. We found that the raft transmembrane protein linker for activation of T cells (LAT), but not a palmitoylation-deficient non-raft LAT mutant, strongly accumulated in TCR-enriched immunoisolates in a tyrosine phosphorylation-dependent manner. In contrast, other raft-associated molecules, including protein tyrosine kinases Lek and Fyn, GM1, and cholesterol, were not highly concentrated in TCR-enriched plasma membrane immunoisolates. Many downstream signaling proteins coisolated with the TCR/LAT-enriched plasma membrane fragments, suggesting that LAT/ TCR assemblies form a structural scaffold for TCR signal transduction proteins. Our results indicate that TCR signaling assemblies in plasma membrane subdomains, rather than generally concentrating raft-associated membrane proteins and lipids, form by a selective protein-mediated anchoring of the raft membrane protein LAT in vicinity of TCR.

3.146 Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid β-protein generation

Xia, W. et al
*Proc. Natl. Acad. Sci., **97(16)**, 9299-9304 (2000)*

An unusual intramembranous cleavage of the β-amyloid precursor protein (APP) by γ-secretase is the final step in the generation of amyloid β-peptide (Aβ). Two conserved aspartates in transmembrane (TM) domains 6 and 7 of presenilin (PS) 1 are required for Aβ production by γ-secretase. Here we report that the APP C-terminal fragments, C83 and C99, which are the direct substrates of γ-secretase, can be coimmunoprecipitated with both PS1 and PS2. PS/C83 complexes were detected in cells expressing endogenous levels of PS. The complexes accumulate when γ-secretase is inactivated either pharmacologically or by mutating the PS aspartates. PS1/ C83 and PS1/C99 complexes were detected in Golgi-rich and trans-Golgi network-rich vesicle fractions. In contrast, complexes of PS1 with APP holoprotein, which is not the immediate substrate of γ-secretase, occurred earlier in endoplasmic reticulum-rich vesicles. The major portion of intracellular Aβ at steady state was found in the same Golgi/trans-Golgi network-rich vesicles, and Aβ levels in these fractions were markedly reduced when either PS1 TM aspartate was mutated to alanine. Furthermore, *de novo* generation of Aβ in a cell-free microsomal reaction occurred specifically in these same vesicle fractions and was markedly inhibited by mutating either TM aspartate. Thus, PSs are complexed with the γ-secretase substrates C83 and C99 in the subcellular locations where Aβ is generated, indicating that PSs are directly involved in the pathogenically critical intramembranous proteolysis of APP.

3.147 Long-term insulin treatment of 3T3-L1 adipocytes results in mis-targeting of GLUT4: implications for insulin-stimulated glucose transport

Maier, V.H. and Gould G.W.

*Diabetologia, **43(10)**, 1273-1281 (2000)*

Aimslhypothesis. Insulin stimulates glucose transport in adipose and muscle tissue by the translocation of a specialised pool of intracellular GLUT4-containing vesicles to the cell surface. It is well established that defective insulin-stimulated GLUT4 translocation is associated with insulin resistance. Long-term insulin treatment (500 nmol/l for 24 h) of 3T3-Ll adipocytes has previously been shown to decrease cellular GLUT4 content and reduce insulin-stimulated GLUT4 translocation. Here, we test the hypothesis that the insulin resistance observed after long-term insulin treatment arises by the selective loss of GLUT4 from a specific intracellular compartment.

Methods Using **iodixanol** gradient centrifugation we have separated intracellular GLUT4 containing membranes into two distinct populations corresponding to recycling endosomes and a distinct intracellular compartment, which probably represents GLUT4 storage vesicles (GSVs).

Results. A short-term insulin stimulation reduced the content of GLUT4 in the GSV fraction (51 ± 3.5 %) with only a modest decrease from the endosomal fraction (23 ± 2.6 %). Long-term insulin treatment decreased cellular GLUT4 content by about 40 % and diminished the ability of a short-term insulin challenge to promote GLUT4 translocation. We further show that this depletion of cellular GLUT4 is selectively from the GSV fraction (68 ± 7 % decrease compared to untreated cells).

Conclusions/interpretation. Such data argue that long-term insulin treatment results in the mistargeting of GLUT4 such that it no longer accesses the GSV compartment. These data imply that defective targeting of GLUT4 away from the GSV compartment plays an important role in the aetiology of insulin resistance.

3.148 Assembly of myelin by association of proteolipid protein with cholesterol and galactosylceramide-rich membrane domains

Simons, M., Kramer, E-M., Thiele, C., Stoffel, W. And Trotter, J.

*J. Cell Biol., **151(1)**, 143-153 (2000)*

Myelin is a specialized membrane enriched in glycosphingolipids and cholesterol that contains a limited spectrum of proteins. We investigated the assembly of myelin components by oligodendrocytes and analyzed the role of lipid-protein interactions in this process. Proteolipid protein (PLP), the major myelin protein, was recovered from cultured oligodendrocytes from a lower-density CHAPS-insoluble membrane fraction (CIMF) enriched in myelin lipids. PLP associated with the CIMF after leaving the endoplasmic reticulum but before exiting the Golgi apparatus, suggesting that myelin lipid and protein components assemble in the Golgi complex. The specific association of PLP with myelin lipids in CIMF was supported by the finding that it was efficiently cross-linked to photoactivable cholesterol, but not to phosphatidylcholine, which is underrepresented in both myelin and CIMF: Furthermore, depletion of cholesterol or inhibition of sphingolipid synthesis in oligodendrocytes abolished the association of PLP with CIMF. Thus PLP may be recruited to myelin rafts, represented by CIMF, via lipid-protein interactions. In contrast to oligodendrocytes, after transfection in BHK cells, PLP is absent from isolated CIMF, suggesting that PLP requires specific lipids for raft association. In mice deficient in the enzyme ceramide galactosyl transferase, which cannot synthesize the main myelin glycophingolipids, a large fraction of PLP no longer associates with rafts. Formation of a cholesterol- and galactosylceramide-rich membrane domain (myelin rafts) may be critical for the sorting of PLP and assembly of myelin in oligodendrocytes.

3.149 Intracellular events in the assembly of chylomicrons in rabbit enterocytes

Cartwright, I.J., Plonne, D. and Higgins, J.A.
*J. Lipid Res., **41**, 1728-1739 (2000)*

The aim of this study was to determine the intracellular events in chylomicron assembly in adult villus enterocytes. We have used novel methods for separation of the intracellular components of the secretory compartment [rough and smooth endoplasmic reticulum (RER and SER), respectively) and Golgi], and their membrane and luminal components, from villus enterocytes isolated from rabbit small intestine. The steady state composition of the components of the secretory compartment and the intracellular pools of newly synthesized. Apolipoprotein B-48 (apoB-48) and triacylglycerol (TAG) was determined. The observations indicate that the SER is the main site of the TAG synthesis and of chylomicron assembly. Newly synthesized apoB-48 and TAG accumulate in the SER membrane and are transferred into the lumen in a microsomal triglyceride transfer protein-dependent step. In enterocytes isolated from chow-fed rabbits, in which fat absorption is relatively slow, transfer of apoB-48 and TAG from the SER membrane into the lumen appears to be rate limiting. In enterocytes from fat-fed rabbits, TAG accumulates into the lumen of the SER, suggesting that movement out of the SER lumen becomes rate limiting, when chylomicron secretion is markedly stimulated. In these cells, the cytosolic TAG also increased to 450 μg/g enterocytes, compared with 12 μg/g enterocytes from chow-fed rabbits, indicating that transfer of TAG from the SER membrane into the secretory pathway can become saturated, so that newly synthesized TAG moves into the cytosol.

3.150 Brain plasmin enhances APP α-cleavage and Aβ degradation and is reduced in Alzheimer's disease brains

Ledesma, M.D., Da Silva, J.S., Crassaerts, K., Delacourte, A., De Strooper, B and Dotti, C.G.
*EMBO Reports, **11(61)**, 530-535 (2000)*

The proteolytic processing of amyloid precursor protein (APP) has been linked to sphingolipid-cholesterol microdomains (rafts). However, the raft proteases that may be involved in APP cleavage have not yet been identified. In this work we present evidence that the protease plasmin is restricted to rafts of cultured hippocampal neurons. We also show that plasmin increases the processing of human APP preferentially at the α-cleavage site, and efficiently degrades secreted amyloidogenic and non-amyloidogenic APP fragments. These results suggest that brain tissue from Alzheimer's disease patients contains reduced levels of plasmin, implying that plasmin downregulation may cause amyloid plaque deposition accompanying sporadic Alzheimer's disease.

3.151 Cytosolic phospholipase A_2 regulates Golgi structure and modulates intracellular trafficking of membrane proteins

Choukroun, G.J. et al
*J. Clin. Invest, **106**, 983-993 (2000)*

The Golgi complex and the *trans*-Golgi network are critical cellular organelles involved in the endocytic and biosynthetic pathways of protein trafficking. Lipids have been implicated in the regulation of membrane protein trafficking vesicular fusion, and targeting. We have explored the role of cytosolic group IV phospholipase A_2 ($cPLA_2$) in membrane-protein trafficking in kidney epithelial cells. Adenoviral expression of $cPLA_2$ in LLC-PK_1 kidney epithelial cells prevents constitutive trafficking to the plasma membrane of an aquaporin 2-green fluorescent protein chimera, with retention of the protein in the rough endoplasmic reticulum. Plasma membrane Na^+-K^+-ATPase α-subunit localization is markedly reduced in cells expressing $cPLA_2$, whereas the trafficking of a Cl^-/ HCO_3^- anion exchanger to the plasma membrane is not altered in these cells. Expression of $cPLA_2$ results in dispersion of giantin and β-COP from their normal, condensed Golgi localization, and in marked disruption of the Golgi cisternae. $cPLA_2$ is present in Golgi fractions from noninfected LLC-PK_1 cells and rat kidney cortex. The distribution of tubulin and actin was not altered by $cPLA_2$ indicating that the microtubule and actin cytoskeleton remain intact. Total cellular protein synthesis is unaffected by the increase in $cPLA_2$ activity. Thus $cPLA_2$ plays an important role in determining Golgi architecture and selective control of constitutive membrane-protein trafficking in renal epithelial cells.

3.152 Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient translocation to the nucleus
Sinzger, C. et al
J. Gen. Virol., ***81****, 3021-3035 (2000)*

Marked interstrain differences in the endothelial cell (EC) tropism of human cytomegalovirus (HCMV) isolates have been described. This study aimed to define the step during the replicative cycle of the HCMV that determines this phenotype. The infection efficiency of various HCMV strains in EC versus fibroblasts was quantified by immunodetection of immediate early (IE), early and late viral antigens. Adsorption and penetration were analyzed by radiolabeled virus binding assays and competitive HCMV-DNA-PCR. The translocation of penetrated viral DNA to the nucleus of infected cells was quantified by competitive HCMV-DNA-PCR in pure nuclear fractions. The intracytoplasmic translocation of capsids that had penetrated was followed by immunostaining of virus particles on a single particle level; this was correlated with the initiation of viral gene expression by simultaneous immunostaining of viral IE antigens. The infectivity of nonendotheliotropic HCMV strains in EC was found to be 100-1000-fold lower when compared to endotheliotropic strains. The manifestation of this phenotype at the level of IE gene expression indicated the importance of initial replication events. Surprisingly, no interstrain differences were detected during virus entry. However, dramatic interstrain differences were found regarding the nuclear translocation of penetrated viral DNA. With nonendotheliotropic strains, the content of viral DNA in the cell nucleus was 100-1000-fold lower in EC when compared to endotheliotropic strains, thereby reflecting the strain differences in IE gene expression. Simultaneous staining of viral particles and viral IE antigen revealed that interstrain differences in the transport of penetrated capsids towards the nucleus of endothelial cells determine the EC tropism of HCMV.

3.153 B cell antigen receptor signaling occurs outside lipid rafts in immature B cells
Sproul, T.W., Malapati, S., Kim, J. and Pierce, S.
J. Immunol., ***165****, 6020-6023 (2000)*

B cell Ag receptor (BCR) signaling changes dramatically during B cell development, resulting in activation in mature B cells and apoptosis, receptor editing, or anergy in immature B cells. BCR signaling in mature B cells was shown to be initiated by the translocation of the BCR into cholesterol- and sphingolipid-enriched membrane microdomains that include the Src family kinase Lyn and exclude the phosphatase CD45. Subsequently the BCR is rapidly internalized into the cell. Here we show that the BCR in the immature B cell line, WEHI-231, does not translocate into lipid rafts following cross-linking nor is the BCR rapidly internalized. The immature BCR initiates signaling from outside lipid rafts as evidenced by the immediate induction of an array of phosphoproteins and subsequent apoptosis. The failure of the BCR in immature B cells to enter lipid rafts may contribute to the dramatic differences in the outcome of signaling in mature and immature B cells.

3.154 Different properties of two isoforms of annexin XIII in MDCK cells
Lecat, S. et al
J. Cell Sci., ***113****, 2607-2618 (2000)*

Annexins form a family of proteins that are widely expressed and known to bind membranes in the presence of calcium. Two isoforms of the annexin XIII subfamily are expressed in epithelia. We previously reported that annexin XIIIb is apically localized in MDCK cells and that it is involved in raft-mediated delivery of apical proteins. We have now analyzed the properties of annexin XIIIa, which differs from annexin XIIIb by a deletion of 41 amino acids in the amino-terminal domain, and is distributed both apically and basolaterally. Annexin XIIIa binding to membranes is independent of calcium but requires its myristoyl amino-terminal modification, as observed with annexin XIIIb. Our biochemical and functional data show that annexin XIIIa behaves differently in the apical and in the basolateral compartments. Whereas annexin XIIIa apically can associate with rafts independently of calcium, the basolateral pool requires calcium for this. Annexin XIIIa, like annexin XIIIb, stimulates apical transport of influenza virus hemagglutinin but, in contrast, only annexin XIIIa inhibits basolateral transport of vesicular stomatitis virus G protein. Our results suggest that annexin XIIIa and XIIIb have specific roles in epithelial cells, and because of their structural similarities, these isoforms offer interesting tools for unravelling the functions of annexins.

3.155 New and re-emerging diseases: A dedication to Norman D. Levine
Docampo, R.
*Parasitology Today, **16(8)**, 316, (2000)*

No abstract

3.156 Differential effects of acyl-CoA binding protein on enzymatic and non-enzymatic thioacylation of protein and peptide substrates
Dunphy, J.T. et al
*Biochem. Biophys. Acta, **1485**, 185-198 (2000)*

Both enzymatic and autocatalytic mechanisms have been proposed to account for protein thioacylation (commonly known as palmitoylation). Acyl-CoA binding proteins (ACBP) strongly suppress non-enzymatic thioacylation of cysteinyl-containing peptides by long-chain acyl-CoAs. At physiological concentrations of ACBP, acyl-CoAs, and membrane lipids, the rate of spontaneous acylation is expected to be too slow to contribute significantly to thioacylation of signaling proteins in mammalian cells (Leventis et al., Biochemistry 36 (1997) 5546-5553). Here we characterized the effects of ACBP on enzymatic thioacylation. A protein S-acyltransferase activity previously characterized using G-protein α-subunits as a substrate (Dunphy et al., J. Biol. Chem., 271 (1996) 7154-7159), was capable of thioacylating short lipid-modified cysteinyl-containing peptides.The minimum requirements for substrate recognition were a free cysteins thiol adjacent to a hydrophobic lipid anchor, either myristate or farnesyl isoprenoid. PAT activity displayed specificity for the acyl donor, efficiently utilizing long-chain acyl-CoAs, but not free fatty acid or S-palmitoyl-*N*-acetylcysteamine. ACBP only modestly inhibited enzymatic thioacylation of a myristoylated peptide or G-protein α-subunits under conditions where non-enzymatic thioacylation was reduced to background. Thus, protein S-acyltransferase remains active in the presence of physiological concentrations of ACBP and acyl CoA in vitro and is likely to represent the predominant mechanism of thioacylation in vivo.

3.157 Cytosolic Hsp70s are involved in the transport of aminopeptidase 1 from the cytoplasm into the vacuole
Satyanarayana, C., Schroder-Kohne, S., Craig, E.A., Schu, P.V. and Horst, M.
*FEBS Lett., **470**, 232-238 (2000)*

Eukaryotic 70 kDa heat shock proteins (Hsp70s) are localized in various cellular compartments and exhibit functions such as protein translocation across membranes, protein folding and assembly. Here we demonstrate that the constitutively expressed members of the yeast cytoplasmic Ssa subfamily, Ssa1/2p, are involved in the transport of the vacuolar hydrolase aminopeptidase 1 from the cytoplasm into the vacuole. The Ssap family members displayed overlapping functions in the transport of aminopeptidase 1. In *SSAI* and *SSAII* deletion mutants the precursor of aminopeptidase 1 accumulated in a dodecameric complex that is packaged in prevacuolar transport vesicles. Ssa1/2p was prominently localized to the vacuolar membrane, consistent with the role we propose for Ssa proteins in the fusion of transport vesicles with the vacuolar membrane.

3.158 Seasonal variation in mussel *Mytilus edulis* digestive gland cytochrome P4501A- and 2E-immunoidentified protein levels and DNA strand breaks (Comet assay)
Shaw, J.P., Large, A.T., Chipman, J.K., Livingstone, D.R. and Peters, L.D.
*Marine Environmental Res., **50**, 405-409 (2000)*

Mytilus edulis digestive gland microsomes were prepared from indigenous populations sampled from a clean reference site (Pot Quin) and an urban-industrial contaminated site (Blackpool) in the UK. Samples were collected in March/April, May, August and December 1998. Western blot analysis was performed using polyclonal antibodies to fish CYP1A and rat CYP2E using partially purified *M. edulis* CYP as a positive control, to aid identification. CYP1A- and CYP2E-immunopositive protein levels showed different site-specific seasonal variation with higher levels of CYP2E determined in May ($P< 0.05$). At both sites, lower levels of CYP1A-immunopositive protein but not CYP2E-immunopositive protein were observed in the samples collected in December ($P < 0.05$). This correlated with lower levels of nuclear DNA damage (Comet assay expressed as per cent tail DNA) observed in December compared to August ($P < 0.05$).

3.159 Tyrosine mutants are capable of prodrug activation in transfected nonmelanotic cells
Simonova, M., Wall, A., Weissleder, R. and Bogdanov, A.
*Cancer Res., **60**, 6656-6662 (2000)*

Tyrosinase has been suggested as a prodrug-converting enzyme for the treatment of melanoma. We hypothesized that tyrosinase expression in transfected nonmelanotic cells can be used in a gene therapy paradigm of prodrug activation. To verify our hypothesis, we used the following tyrosinase variants: (a) a full-length human tyrosinase clone (T); (b) a mutant lacking the COOH-terminal cytoplasmic domain (TΔC); (c) a mutant lacking the COOH-terminal transmembrane and cytoplasmic domains (TΔTC); and (d) a fusion with the eight COOH-terminal amino acids of lysosome-associated membrane protein-1 (TL). Expression of mutant and wild-type tyrosinases was induced by transfection in nontumorigenic human cells of epithelial origin (293HEK, MCF-10A adenoma, and NHDF-Ad human dermal fibroblasts) as well as in tumour cells (9L gliocarcinoma, MCF7 adenocarcinoma and HT-1080 fibrosarcoma). When compared with the wild-type tyrosinase transfectants, truncated mutant expression resulted in higher mRNA levels that paralleled higher enzyme activity of the truncated mutants. Two model tyrosinase prodrugs, hydroxyphenyl-propanol (HPP) and N-acetyl-4-S-cysteaminylphenol (NAcSCAP) inhibited proliferation and caused cell death of transfected cells in a dose-dependent manner. Effects of prodrug treatment were compared for tumorigenic cells and their nontumorigenic counterparts. Two truncated mutants (TΔC and TΔTC) showed low endogenous cytotoxicity and efficiently suppressed proliferation and induced cytotoxicity in transfected tumor cells in the presence of NAcSCAP. Overall, these results indicate that the developed tyrosinase mutants hold promise as prodrug activation systems for tumoral gene therapy.

3.160 GFRα –mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival
Tansey, M.G., Baloh, R.H., Milbrandt, J. and Johnson, Jr., E.M.
*Neuron, **25**, 611-623 (2000)*

The GDNF family ligands (GFLs: GDNF, neurturin, persephin, and artemin) signal through RET and a glycosyl-phosphatidylinositol (GPI)-anchored coreceptor (GFRα1-α4) that binds ligand with high affinity and provides specificity. The importance of the GPI anchor is not fully understood; however, GPI-linked proteins cluster into lipid rafts, structures that may represent highly specialized signaling organelles. Here, we report that GPI-anchored GFRα 1 recruits RET to lipid rafts after GDNF stimulation and results in RET/Src association. Disruption of RET localization using either transmembrane-anchored or soluble GFRα1 results in RET phosphorylation, but GDNF-induced intracellular signaling events are markedly attenuated as are neuronal differentiation and survival responses. Therefore, proper membrane localization of RET via interaction with a raft-localized, GPI-linked coreceptor is of fundamental importance in GFL signaling.

3.161 Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease
Takahashi, M. et al
*Neuron, **28**, 461-473 (2000)*

Amyloid precursor protein (APP) generates the β-amyloid peptide, postulated to participate in the neurotoxicity of Alzheimer's disease. We report that APP and APLP bind to heme oxygenase (HO), an enzyme whose product, bilirubin, is antioxidant and neuroprotective. The binding of APP inhibits HO activity, and APP with mutations linked to the familial Alzheimer's disease (FAD) provides substantially greater inhibition of HO activity than wild-type APP. Cortical cultures from transgenic mice expressing Swedish mutant APP have greatly reduced bilirubin levels, establishing that mutant APP inhibits HO activity in vivo. Oxidative neurotoxicity is markedly greater in cerebral cortical cultures from APP Swedish mutant transgenic mice than wild-type cultures. These findings indicate that augmented neurotoxicity caused by APP-HO interactions may contribute to neuronal cell death in Alzheimer's disease.

3.162 Mutational and biochemical analysis of plasma membrane targeting mediated by the farnesylated, polybasic carboxy terminus of K-ras4B

Roy, M-O., Leventis, R. and Silvius, J.R.

*Biochemistry, **39**, 8298-8307 (2000)*

Mutational analysis and in vitro assays of membrane association have been combined to investigate the mechanism of plasma membrane targeting mediated by the farnesylated, polybasic carboxy-terminal sequence of K-ras4B in mammalian cells. Fluorescence-microscopic localization of chimeric proteins linking the enhanced green fluorescent protein (EGFP) to the K-ras4B carboxy-terminal sequence, or to variant forms of this sequence, reveals that the normal structure of this targeting motif can be greatly altered without compromising plasma membrane-targeting activity so long as an overall strongly polybasic/amphiphilic character is retained. An EGFP/K-ras4B(171-188) chimeric protein was readily abstracted from isolated cell membranes by negatively charged lipid vesicles, and this abstraction was markedly enhanced by the anionic lipid-binding agent neomycin. Our results strongly favor a mechanism in which at the plasma membrane the carboxy-terminal sequence of K-ras4B associates not with a classical specific proteinaceous receptor but rather with nonspecific but highly anionic 'sites' formed at least in part by the membrane lipid bilayer. Our findings also suggest that the recently demonstrated prenylation-dependent trafficking of immature forms of K-ras4B through the endoplasmic reticulum [Choy et al. (1999) Cell 98, 69-80], while required for maturation of the protein, beyond this stage may not be essential to allow the ultimate delivery of the mature protein to the plasma membrane.

3.163 An ever-expanding story of cyst formation

Gallagher, A.R., Obermüller, N., Cedzich, A., Gretz, N. and Witzgall, R.

*Cell Tissue Res., **300**, 361-371 (2000)*

Autosomal-dominant polycystic kidney disease represents one of the most common monogenetic human disorders. The cloning of the *PKD1* and *PKD2* genes, which are mutated in far more than 90% of the patients affected by this disease, has generated high hopes for a quick understanding of the pathogenesis of cyst formation. However, these expectations have not yet been fulfilled, since the function of both polycystin-1 and polycystin-2, the two proteins encoded by *PKD1* and *PKD2*, still remains a puzzle. In this review, we will highlight some of the characteristics of polycystic kidney disease, briefly touch on polycystin-1, and then go on to describe recent results of experiments with polycystin-2, since the latter is the major focus of our work. We will discuss new evidence which suggests that autosomal-dominant polycystic kidney disease actually behaves recessively on a cellular level. Finally, a model will be presented that tries to explain the available data.

3.164 Ultrastructural characterization of the delimiting membranes of isolated autophagosomes and amphisomes by freeze-fracture electron microscopy

Fengsrud, M., Erichsen, E.S., Berg, T.O., Raiborg, C. And Seglen, P.O.

*Eur. J. Cell Biol., **79**, 871-882 (2000)*

The delimiting membranes of isolated autophagosomes from rat liver had extremely few transmembrane proteins, as indicated by the paucity of intramembrane particles in freeze-fracture images (about 20 particles/um^2, whereas isolated lysosomes had about 2000 particles/um^2). The autophagosomes also appeared to lack peripheral surface membrane proteins, since attempts to surface-biotinylate intact autophagosomes only yielded biotinylation of proteins from contaminating damaged mitochondria. All the membrane layers of multilamellar autophagosomes were equally particle-poor; the same was true of the autophagosome-forming, sequestering membrane complexes (phagophores). Isolated amphisomes (vacuoles formed by fusion between autophagosomes and endosomes) had more intramembrane particles than the autophagosomes (about 90 particles/um^2), and freeze-fracture images of these organelles frequently showed particle-rich endosomes fusing with particle-poor or particle-free autophagosomes. The appearance of multiple particle clusters suggested that a single autophagic vacuole could undergo multiple fusions with endosomes. Only the outermost membrane of bi-or multilammelar autophagic vacuoles appeared to engage in such fusions.

3.165 The phagacytosis-associated respiratory burst in human monocytes is associated with increased uptake of glutathione

Seres, T., Knickelbein, R.G., Warshaw, J.B. and Johnston, Jr, R.B.
J. Immunol., ***165****, 3333-3340 (2000)*

During the phagocytic respiratory burst, oxygen is converted to potent cytotoxic oxidants. Monocytes and macrophages are potentially long-lived, and we have hypothesized that protective mechanisms against oxidant stress are varied and fully expressed in these cells. We report here that the respiratory burst in monocytes is accompanied by an increase in the uptake of [^{35}S]glutathione ([^{35}S]GSH) after 20–30 min to levels up to 10-fold greater than those at baseline. By 30 min, 49% of the cell-associated radioactivity was in the cytosol, 41% was in membrane, and 10% was associated with the nuclear fraction. GSH uptake was inhibited by catalase, which removes hydrogen peroxide (H_2O_2), and micromolar H_2O_2 stimulated GSH uptake effectively in monocytes and also lymphocytes. Oxidation of GSH to glutathione disulfide with H_2O_2 and glutathione peroxidase prevented uptake. Acivicin, which inhibits GSH breakdown by γ-glutamyl transpeptidase (GGT), had no effect on the enhanced uptake seen during the respiratory burst. Uptake of cysteine or cystine, possible products of GGT activity, stayed the same or decreased during the respiratory burst. These results suggest that a GGT-independent mechanism is responsible for the enhanced GSH uptake seen during the respiratory burst. We describe here a sodium-independent, methionine-inhibitable transport system with a K_m (8.5 μM) for GSH approximating the plasma GSH concentration. These results suggest that monocytes have a specific GSH transporter that is triggered by the release of H_2O_2 during the respiratory burst and that induces the uptake of GSH into the cell. Such a mechanism has the potential to protect the phagocyte against oxidant damage.

3.166 Expression and localization of rab escort protein isoforms in parotid acinar cells from rat

Chan, D., Lin, J. and Raffaniello, R.D.
J. Cell. Physiol., ***185(3)****, 339-347 (2000)*

Rab proteins are geranylgeranylated on their carboxyl terminal cysteine motifs by geranylgeranyltransferase II (GGTase). Rab escort protein (REP) is required to present Rab proteins to GGTase. REP may remain bound to newly isoprenylated Rab proteins and present them to their target membrane. Other studies have shown that Rab proteins cycle between the membrane and cytosolic compartments and that cytosolic Rab proteins are complexed with rab-GDI. In the present study, we examined the expression and localization of REP isoforms in parotid acinar cells. Although both REP isoforms, REP-1 and REP-2, were detected in parotid cytosol, REP-2 was the predominant isoform. Subcellular fractionation revealed that approximately 42% of cellular REP-2 is membrane-associated. REP-2 was partially removed from parotid membranes with 1 M NaCl or Na2CO3, indicating that REP-2 is a peripheral membrane protein. Membrane-associated REP-2 did not colocalize with Rab3D on secretory granule membranes. However, density gradient centrifugation revealed that membrane-associated REP-2 and Rab3D colocalize on low- and high-density membrane fractions in parotid acinar cells. Isoproterenol, an agent which induces amylase release from parotid glands, caused a shift in both REP-2 and Rab3D to less dense membrane fractions. When acinar cell cytosol was fractionated by gel filtration chromatography, Rab3D eluted exclusively with REP, not rab-GDI. In contrast, Rab1B and Rab5 eluted with both REP and Rab-GDI. Colocalization of Rab3D and REP-2 on acinar cell membranes suggests that REP-2 plays a role in delivering Rab3D to parotid membranes and may regulate guanine nucleotide binding to membrane-associated Rab3D. In addition, unlike other Rab proteins, cytosolic Rab3D appears to associate exclusively with REP, not rab-GDI in parotid acinar cells.

3.167 Mutation of conserved aspartates affect maturation of presenilin 1 and presenilin 2 complexes

Yu, G., Chen, F., Nishimura, M., Steiner, H., Tandon, A., Kawarai, T., Arawaka, S., Supala, A., Song, Y-Q., Rogaeva, E., Holmes, E., Zhang, D.M., Milman, P., Fraser, P., Haass, C. and St. George-Hyslop, P.
Acta Neurol Scand., ***Suppl. 176****, 6-11 (2000)*

Presenilin (PS1 and PS2) holoproteins are transiently incorporated into low molecular weight (MW) complexes. During subsequent incorporation into a higher MW complex, they undergo endoproteolysis to generate stable N- and C-terminal fragments (NTF/CTF). Mutation of either of two conserved aspartate residues in transmembrane domains inhibits both presenilin-endoproteolysis and the proteolytic processing of APP and Notch. We show that aspartate-mutant holoprotein presenilins are not incorporated into the high molecular weight, NTF/CTF-containing complexes. Aspartate-mutant presenilin holoproteins also preclude entry of endogenous wild-type PS1/PS2 into the high molecular weight complexes, but do not affect the incorporation of wild-type holoproteins into lower molecular weight holoprotein complexes.

These data suggest that the loss-of-function aspartate-mutants cause altered PS complex maturation, and argue that the functional presenilin moieties are contained in the high molecular weight presenilin NTF/CTF-containing complexes.

3.168 Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase
Teter, S.A. et al
J. Biol. Chem., ***276(1)***, *2083-2087 (2001)*

The vacuole/lysosome serves an essential role in allowing cellular components to be degraded and recycled under starvation conditions. Vacuolar hydrolases are key proteins in this process. In *Saccharomyces cerevisiae*, some resident vacuolar hydrolases are delivered by the cytoplasm to vacuole targeting (Cvt) pathway, which shares mechanistic features with autophagy. Autophagy is a degradative pathway that is used to degrade and recycle cellular components under starvation conditions. Both the Cvt pathway and autophagy employ double-membrane cytosolic vesicles to deliver cargo to the vacuole. As a result, these pathways share a common terminal step, degradation of subvacuolar vesicles. We have identified a protein, Cvt17, which is essential for this membrane lytic event. Cvt17 is a membrane glycoprotein that contains a motif conserved in esterases and lipases. The active-site serine of this motif is required for sub-vacuolar vesicle lysis. This is the first characterization of a putative lipase implicated in vacuolar function in yeast.

3.169 An ephrinA-dependent signaling pathway controls integrin function and is linked to the tyrosine phosphorylation of a 120 kDa protein
Huai, J.and Drescher, U.
J. Biol. Chem., ***276(9)***, *6689-6694 (2001)*

The Eph family of receptor tyrosine kinases and their ligands, the ephrins, have been implicated in the development of the retinotectal projection. Here, glycosylphoaphatidylinositol-anchored A-ephrins are not only expressed in the tectum, but also on retinal axons, raising the possibility that they function in this context as receptors. We now show that activation of ephrinA2 or ephrinA5 by one of their receptors, ephA3, results in a β1-integrin dependent increased adhesion of ephrinA-expressing cells to laminin. In the search for an ephrinA-dependent signaling pathway controlling integrin activation, we identified a 120 kDa raft membrane protein which is tyrosine phosphorylated specifically after ephrinA activation. Tyrosine phosphorylation of this protein is not seen after stimulating ephrinA2-expressing cells with basic fibroblast growth factor, epidermal growth factor, insulin growth factor or fetal calf serum containing a large set of different growth factors. The role of p120 as a mediator of an ephrinA - integrin coupling is supported by the finding that inhibiting tyrosine phosphorylation of pl20 correlates with an abolishment of the β1-dependent cell adhesion.

3.170 The synaptic vesicle protein, cysteine-string, is associated with the plasma membrane in 3T3-L1 adipocytes and interacts with syntaxin 4
Chamberlain, L.H. et al
J. Cell Sci., ***114(2)***, *445-455 (2001)*

Adipocytes and muscle cells play a major role in blood glucose homeostasis. This is dependent upon the expression of Glut4, an insulin-responsive facilitative glucose transporter. Glut4 is localized to specialized intracellular vesicles that fuse with the plasma membrane in response to insulin stimulation. The insulin-induced translocation of Glut4 to the cell surface is essential for the maintenance of optimal blood glucose levels, and defects in this system are associated with insulin resistance and type II diabetes. Therefore, a major focus of recent research has been to identify and characterize proteins that regulate Glut4 translocation. Cysteine-string protein (Csp) is a secretory vesicle protein that functions in presynaptic neurotransmission and also in regulated exocytosis from non-neuronal cells. We show that Csp1 is expressed in 3T3-L1 adipocytes and that cellular levels of this protein are increased following cell differentiation. Combined fractionation and immunofluorescence analyses reveal that Csp1 is not a component of intracellular Glut4-storage vesicles (GSVs), but is associated with the adipocytes plasma membrane. This association is stable, and not affected by either insulin stimulation or chemical depalmitoylation of Csp1. We also demonstrate that Csp1 interacts with the t-SNARE syntaxin 4. As syntaxin 4 is an important mediator of insulin-stimulated GSV fusion with the plasma membrane, this suggests that Csp1 may play a regulatory role in this process. Syntaxin 4 interacts specifically with Csp1, but not with Csp2. In contrast, syntaxin 1A binds to both Csp isoforms, and actually exhibits a higher affinity for the Csp2 protein.

The results described raise a number of interesting questions concerning the intracellular targeting of Csp in different cell types, and suggest that the composition and synthesis of GSVs may be different from synaptic and other secretory vesicles. In addition, the interaction of Cps1 with syntaxin 4 suggests that this Csp isoform may play a role in insulin-stimulated fusion of GSVs with plasma membrane.

3.171 Pro-caspase-8 is pre-dominantly localized in mitochondria and released into cytoplasm upon apoptotic stimulation
Qin, Z-H. et al
J. Biol. Chem., ***276(11)****, 8079-8086 (2001)*

The recruitment and cleavage of pro-caspase-8 to produce the active form of caspase-8 is a critical biochemical event in death receptor-mediated apoptosis. However, the source of pro-caspase-8 available for activation by apoptotic triggers is unknown. In human fibroblasts and mouse clonal striatal cells, confocal microscopy revealed that pro-caspase-8 immunofluorescence was co-localized with cytochrome c in mitochondria and was also distributed diffusely in some nuclei. Biochemical analysis of subcellular fractions indicated that pro-caspase-8 was enriched in mitochondria and in the nucleus. Pro-caspase-8 was found in the intermembrane space, inner membrane and matrix of mitochondria after limited digestion of mitochondrial fractions and this distribution was confirmed by immunogold electron microscopy. Pro-caspase-8 and cytochrome c were released from isolated mitochondria that were treated with an inhibitor of the ADP/ATP carrier atractyloside (Atr), which opens the mitochondrial permeability transition pore. Release was blocked by the mitochondrial permeability transition pore inhibitor cyclosporin A (CsA). After clonal striatal cells were exposed for 6 hr to the apoptotic inducer, tumor necrosis factor-α (TNF-α), mitochondria immunoreactive for cytochrome c and pro-caspase-8 became clustered at perinuclear sites. Pro-caspase-8 and cytochrome c levels decreased in mitochondrial fractions and increased, along with pro-caspase-8a cleavage products, in the cytoplasm of the TNF-alpha treated striatal cells. CsA blocked the TNF-α-induced release of pro-caspase 8, but not cytochrome c. Internucleosomal DNA fragmentation started at 6 hr and peaked at 12 hr after TNF-α treatment. These results suggest that pro-caaspase-8 is predominately localized in mitochondria and is released upon apoptotic stimulation through a CsA-sensitive mechanism.

3.172 Hrs interacts with sorting nexin 1 and regulates degradation of epidermal growth factor receptor
Chin, L-S., Raynor, M.C., Wei, X., Chen, H-Q. and Li, L.
J. Biol. Chem., ***276(10)****, 7069-7078 (2001)*

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is a mammalian homologue of yeast vacuolar protein sorting (Vps) protein Vps27p, however the role of Hrs in lysosomal trafficking is unclear. Here, we report that Hrs interacts with sorting nexin 1 (SNX1), a recently identified mammalian homologue of yeast Vps5p that recognize the lysosomal targeting code of epidermal growth factor receptor (EGFR) and participates in lysosomal trafficking of the receptor. Biochemical analyses demonstrate that Hrs and SNX1 are ubiquitous proteins that exist in both cytosolic and membrane-associated pools, and that the association of Hrs and SNX occurs on cellular membranes but not in the cytosol. Furthermore, endogenous SNX1 and Hrs form a ~550-kDa complex that excludes EGFR. Immunofluorescence and subcellular fractionation studies show that Hrs and SNX1 colocalize on early endosomes. By using depletion analysis, we have mapped the binding domains of Hrs and SNX1 that mediate their association. Overexpression of Hrs or its SNX1-binding domain inhibits ligand-induced degradation of EGFR, but does not affect either constitutive or ligand-induced receptor-mediated endocytosis. These results suggest that Hrs may regulate lysosomal trafficking through its interaction with SNX1.

3.173 Localization and insulin-regulated relocation of phosphoinositide 5-kinase PIKfyve in 3T3-L1 adipocytes
Shisheva, A., Rusin, B., Ikonomov, O.C., DeMarco, C. and Sbrissa, D.
J. Biol. Chem., ***276(15)****, 11859-11869 (2001)*

The mammalian phosphoinositide kinase PIKfyve catalyzes the synthesis of phosphatidylinositol 5-P and phosphatidylinositol 3,5-P_2, thought essential in cellular functions, including membrane trafficking. To discern the intracellular loci of PIKfyve product's formation, we have examined the localization of PIKfyve protein vs. enzymatic activity, and a possible acutely regulated redistribution in 3T3-L1 adipocytes. Subcellular fractions of resting cells that were positive for immunoreactive PIKfyve, such as cytosol (~76%), internal structures (LDM, composed of recycling endosomes, GLUT4 storage compartment, Golgi and cytoskeletal elements) (~20%), and plasma membrane (~4%), expressed

enzymatically active PIKfyve. While presence of FYVE finger in PIKfyve predicts early endosome targeting, density gradient sedimentation, immunoadsorption and fluorescence microscopy analyses segregated the LDM-associated PIKfyve from the membranes of the recycling endosomes and GLUT4. PIKfyve fluorescence staining largely coincided with trans-Golgi network/multivesicular body markers, indicating PIKfyve's role in the late endocytic/biosynthetic pathways. A subfraction of particulate PIKfyve resisted nonionic detergent treatment, implying association with cytoskeletal structures, previously found positive for key members of the insulin signaling cascade. Upon acute stimulation of 3T3-L1 adipocytes with insulin or pervanadate, a portion of the cytosolic PIKfyve was recruited onto LDM, which was coupled with a commensurate increase of PIKfyve lipid kinase activity and an electrophoretic mobility shift. We suggest the recruited PIKfyve specifies the site and timing of phosphoinositide signals that are relevant to the acute insulin action.

3.174 Active (9.6S) and inactive (21S) oligomers of NHE3 in distinct microdomains of the renal brush border

Biemesderfer, D., DeGray, B. and Aronson, P.S.
J. Biol. Chem., ***276(13)***, *10161-10167* ***(****2001)*

We have previously shown that Na^+-H^+ exchanger isoform NHE3 exists as both 9.6 S and 21 S (megalin-associated) oligomers in the renal brush border (Biesmesderfer et al., JBC 274, 17518, 1999). To characterize the oligomeric forms of the renal brush border Na^+-H^+ exchanger in more detail, we performed membrane fractionation studies. We found that similar amounts of NHE3 were present in microvilli and a non-microvillar membrane domain of high density (dense vesicles). Horseradish peroxidase-labeled endosomes were not prevalent in the dense membrane fraction. However, megalin, which localizes primarily to the intermicrovillar microdomain of the brush border, was enriched in the dense vesicles, implicating this microdomain as the likely source of these membranes. Immunolocalization of NHE3 confirmed that a major fraction of the transporter co-localized with megalin in the intermicrovillar region of the brush border. Immunoprecipitation studies demonstrated that in microvilli the majority of NHE3 was not bound to megalin, while in the dense vesicles most of the NHE3 co-precipitated with megalin. Moreover, sucrose velocity gradient centrifugation experiments revealed that most NHE3 in microvilli sedimented with an S-value of 9.6 while the S-value of NHE3 in dense vesicles was 21. Finally, we examined the functional state of NHE3 in both membrane fractions. As assayed by changes in acridine orange fluorescence, imposing an outwardly directed Na^+ gradient caused generation of an inside-acid pH gradient in the microvilli, indicating Na^+-H^+ exchange activity, but not in the dense vesicles. Taken together, these data demonstrate that renal brush border NHE3 exists in two oligomeric states; a 9.6 S active form present in microvilli, and a 21 S, megalin-associated, inactive form in the intermicrovillar microdomain of the apical plasma membrane. Thus, regulation of renal brush border Na^+-H^+ exchange activity may be mediated by shifting the distribution between these forms of NHE3.

3.175 Membrane lipid rafts are necessary for the maintenance of the α7 nicotinic acetylcholine receptor in somatic spines of ciliary neurons

Bruses, J.L., Chauvet, N. and Rutishauser, U.
J. Neurosci., ***21(2)***, *504-512 (2001)*

Calcium-permeable neurotransmitter receptors are concentrated into structurally and biochemically isolated cellular compartments to localize calcium-mediated events during neurotransmission. The cytoplasmic membrane contains lipid microdomains called lipid rafts, which can gather into microscopically visible clusters, and thus the association of a particular protein with lipid rafts can result in its redistribution on the cell surface. The present study asks whether lipid rafts participate in the formation and maintenance of the calcium-permeable α7-subunit nicotinic acetylcholine receptor (α7nAChR) clusters found in somatic spines of ciliary neurons. Lipid rafts and α7nAChR become progressively colocalized within somatic spines during synaptogenesis. To determine whether these rafts are required for the maintenance of a7nAChR aggregates, cholesterol was extracted from dissociated ciliary neurons by treatment with methyl-β-cyclodextrin. This treatment caused the dispersion of lipid rafts and the redistribution of α7nAChR into small clusters over the cell surface, suggesting that the integrity of lipid rafts is required to maintain the receptor clustering. However, lipid raft dispersion also caused the depolymerization of the F-actin cytoskeleton, which can also tether the receptor at specific sites. To assess whether interaction between rafts and α7nAChR is independent of F-actin filaments, the lipid raft patches were stabilized with a combination of the cholera toxin B subunit (CTX), which specifically binds to the raft component ganglioside GM1, and an antibody against CTX. The stabilized rafts were then treated with latrunculin-A to depolymerize F-actin. Under these conditions, large patches of CTX persisted and

were colocalized with a7nAChR, indicating that the aggregates of receptors can be maintained independently of the underlying F-actin cytoskeleton. Moreover, it was found that the a7nAChR is resistant to detergent extraction at 4^0C and floats with the caveolin-containing lipid-rich fraction during density gradient centrifugation, properties that are consistent with a direct association between the receptor and the membrane microdomains.

3.176 Mutations in sialidosis impair sialidase binding to the lysosomal multienzyme complex
Lukong, K.E. et al
*J. Biol. Chem., **276,** 17286-17290 (2001)*

Sialidosis is an autosomal recessive disease caused by the genetic deficiency of lysosomal sialidase, which catalyzes the catabolism of sialoglycoconjugates. The disease is associated with progressive impaired vision, macular cherry-red spots, and myoclonus (sialidosis type 1) or with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation, and hepatosplenomegaly (sialidosis type II). We have analyzed the effect of missense mutations, G68V, Sl82G, G227R, F26OY, L270F, A298V, G328S and L363P, identified in the sialidosis type I and sialidosis type II patients on the activity, stability and intracellular distribution of the sialidase. We found that 3 mutations, F260Y, L270F and A298V which are clustered in the same region on the surface of sialidase molecule dramatically reduce the enzyme activity and cause a rapid intralysosomal degradation of the expressed protein. We suggested that this region might be involved in the sialidase binding with lysosomal cathepsin A and/or β-galactosidase in the multienzyme lysosomal complex required for the expression of sialidase activity. Transgenic expression of mutants followed by density gradient centrifugation of cellular extracts confirmed this hypothesis and showed that sialidase deficiency in some sialidosis patients results from disruption of the lysosomal multienzyme complex.

3.177 Mammalian sprouty-1 and –2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells

Impagnatiello, M-A. et al

*J. Cell Biol., **152(5)**, 1087-1098 (2001)*

Growth factor-induced signaling by receptor tyrosine-kinases (RTKs) plays a central role in embryonic development and in pathogenesis and, hence, is tightly controlled by several regulatory proteins. Recently, Sprouty, an inhibitor of *Drosophila* development-associated RTK signaling, has been discovered. Subsequently, four mammalian Sprouty homologues (Spry 1-4) have been identified. Here, we report for functional characterization of two of them, Spry-1 and –2, in endothelial cells. Overexpressed Spry-1 and -2 inhibit fibroblast growth factor- and vascular endothelial growth factor-induced proliferation and differentiation by repressing pathways leading to p42/44 mitogen activating protein (MAP) kinase activation. In contrast, although epidermal growth factor-induced proliferation of endothelial cells were also inhibited by Spry-1 and –2, activation of p42/44 MAP kinase was not affected. Biochemical and immunofluorescence analysis of endogenous and overexpressed Spry-1 and –2 reveal that both Spry-1 and –2 are anchored to membranes by palmitoylation and associate with caveolin-1 in perinuclear and vesicular structures. They are phosphorylated on serine residues and, upon growth factor stimulation, a subset is recruited to the leading edge of the plasma membrane. The data indicate that mammalian Spry-1 and -2 are membrane-anchored proteins that negatively regulate angiogenesis-associated RTK signaling, possibly in an RTK-specific fashion.

3.178 Cytosolic phospholipase A_2-α associates with plasma membrane, endoplasmic reticulum and nuclear membrane in glomerular epithelial cells

Liu, J., Takano, T., Papillon, J., Khadir, A. and Cybulsky, A.V.

*Biochem. J., **353**, 79-90 (2001)*

Eicosanoids mediate complement-dependent glolmerular epithelial injury in experimental membranous nephropathy. The release of arachidonic acid from phospholipids by cytosolic phospholipase A_2 ($cPLA_2$) is the rate-limiting step in eicosanoid synthesis. The present study examines the association of $cPLA_2$ with membranes of organelles. Glomerular epithelial cells were disrupted by homogenization in Ca^{2+} -free buffer; organelles were separated by gradient centrifugation. The distribution of $cPLA_2$, and organelles was analyzed by immunoblotting with antibodies against $cPLA_2$ and organelle markers, or by enzyme assay. In cells incubated with or without the Ca^{2+} ionophore ionomycin plus PMA, $cPLA_2$ co-localized with plasma membrane, endoplasmic reticulum and nuclei, but not with mitochondria or Golgi. A greater amount of $cPLA_2$ was associated with membranes in stimulated cells, but membrane-associated $cPLA_2$ was readily detectable under resting conditions. The pattern of association of $cPLA_2$ with membrane in cells treated with antibody and complement was similar to that in cells stimulated with ionomycin plus PMA; however, complement did not enhance the membrane association of $cPLA_2$ protein. To determine the functional role of membrane association of $cPLA_2$, phospholipids were labeled with [^{3}H]arachidonic acid. Cells were then incubated with or without antibody and complement and were fractionated. Complement induced a loss of radioactivity from the plasma membrane, endoplasmic reticulum and nuclei, but not from the mitochondrial fraction. Thus the release of arachidonic acid by $cPLA_2$ is due to the hydrolysis of phospholipids at multiple subcellular membrane sites, including the endoplasmic reticulum, plasma membrane and nucleus.

3.179 Raft-partitioning of the ubiquitin ligases CbI and Nedd4 upon IgE-triggered cell signaling
Lafont, F. and Simons, K.
*Proc. Natl. Acad. Sci., USA., **98**; 3180-3184 (2001).*

The high affinity receptor for IgE, FcεRI on mast calls and basophils plays an essential role in immunological defense. Upon multivalent antigen binding, FcεRI becomes phoshorylated by the protein--tyrosine kinase Lyn, as a result of receptor clustering in lipid rafts. FcεRI has been shown to be ubiquitinated. Ubiquitination can lead to degradation by proteasomes, but it can also act as a sorting signal to internalize proteins destined to the endosomal/lysosomal pathway. We have analyzed whether FcεRI ubiquitination takes place within rafts. We report biochemical and imaging evidence in rat basolukemia cells for the presence of ubiquitinated FcεRI in clustered rafts upon receptor activation. Moreover, we demonstrated that the ubiquitin ligases CbI and Nedd4 co-localize with FcεRI patches and showed that both ligases become assoc-iated with lipid rafts after activation of IgE signaling. Because CbI is known to interact with the FcεRI signaling complex, ubiquitination is likely to be an important parameter regulating IgE-triggered signaling occurring in rafts.

3.180 Cell-specific targeting of caveolin-1 to caveolae, secretory vesicles, cytoplasm or mitochondria
Li, W-P et al
*J. Cell Sci., **114(7)**, 1397-1408 (2001)*

In commonly used tissue culture cells, caveolin-1 is embedded in caveolae membranes. It appears to reach this location after being cotranslationally inserted into ER membranes, processed in the Golgi and shipped to the cell surface. We now report that caveolae are not the preferred location for caveolin-1 in all cell types. Skeletal muscle cells and keratinocytes target caveolin-1 to the cytosol while in exocrine and endocrine cells it accumulates in the secretory pathway. We also found that airway epithelial cells accumulate cevaolin-1 in modified mitochondria. The cytosolic and the secreted forms appear to be incorporated into a soluble, lipid complex. We conclude that caveolin-1 can be targeted to a variety of intracellular destinations, which suggests a novel mechanism for the intracellular traffic of this protein.

3.181 Glycolipid antigen processing for presentation by CD1d molecules
Prigozy, T.I. et al
*Science, **291**, 664-667 (2001)*

The requirement for processing glycolipid antigens in T cell recognition was examined with mouse CD1d-mediated responses to glycosphingolipids (GSLs). Although some disaccharide GSL antigens can be recognized without processing, the responses to three other antigens, including the disaccharide GSL Gal(α1$\rightarrow$2)GalCer (Gal, galactose; GalCer, galactosylceramide), required removal of the terminal sugars to permit interaction with the T cell receptor. A lysosomal enzyme, α-galactosidase A, was responsible for the processing of Gal(α1$\rightarrow$2)GalCer to generate the antigenic monosaccharide epitope. These data demonstrate a carbohydrate antigen processing system analogous to that used for peptides and an ability of T cells to recognize processed fragments of complex glycolipids.

3.182 Segregation of heterotrimeric G proteins in cell surface microdomains
Oh, P. and Schnitzer, J.E.
*Mol. Biol. Cell, **12**, 685-698 (2001)*

Select lipid-anchored proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins and nonreceptor tyrosine kinase may preferentially partition into sphingomyelin-rich and cholesterol-rich plasmalemmal microdomains, thereby acquiring resistance to detergent extraction. Two such domains, caveolae and lipid rafts, are morphologically and biochemically distinct, contain many signaling molecules, and may function in compartmentalizing cell surface signaling. Subfractionation and confocal immunofluorescence microscopy reveal that, in lung tissue and in cultured endothelial and epithelial cells, heterotrimeric G proteins (G_i, G_q, G_s, and $G_{\beta\gamma}$) target discrete cell surface microdomains. G_q specifically concentrates in caveolae, whereas G_i and G_s concentrate much more in lipid rafts marked by GPI-anchored proteins (5' nucleotidase and folate receptor). G_q, apparently without $G_{\beta\gamma}$ subunits, stably associates with plasmalemmal and cytosolic caveolin. G_i and G_s interact with $G_{\beta\gamma}$ subunits but not caveolin.G_i and G_s unlike G_q, readily move out of caveolae. Thus, caveolin may function as a scaffold to trap, concentrate, and stabilize G_q preferentially within caveolae over lipid rafts. In N2a cells lacking caveolae and caveolin, G_q, G_i, and G_s all concentrate in lipid rafts as a complex with $G_{\beta\gamma}$. Without effective physiological interaction

with caveolin, G proteins tend by default to segregate in lipid rafts. The ramifications of the segregated microdomain distribution and the G_q-caveolin complex without $G_{\beta\gamma}$ for trafficking, signaling, and mechanotransduction are discussed.

3.183 Depletion of rafts in late endocytic membranes is controlled by NPC1-dependent recycling of cholesterol to the plasma membrane
Lusa, S., et al
J. Cell Sci., ***114(10),*** *1893-1900 (2001)*

In mammalian cells, cholesterol is thought to associate with sphingolipids to form lateral membrane domains termed rafts. Increasing evidence suggests that rafts regulate protein interactions, for example, during signaling, intracellular transport and host-pathogen interactions. Rafts are present in cholesterol-sphingolipid-enriched membranes, including early and recycling endosomes, but whether rafts are found in late endocytic organelles has not been analyzed. In this study, we analyzed the association of cholesterol and late endosomal proteins with low-density detergent-resistant membranes (DRMs) in normal cells and in cells with lysosomal cholesterol-sphingolipid accumulation. In normal cells, the majority of [^{3}H]cholesterol released from [^{3}H]cholesterol ester-LDL associated with detergent-soluble membranes, was rapidly transported to the plasma membrane and became increasingly insoluble with time. In Niemann-Pick C1 (NPC1) protein deficient lipidosis cells, the association of LDL-cholesterol with DRMs was enhanced and its transport to the plasma membrane was inhibited. In addition, the NPC1 protein was normally recovered in detergent-soluble membranes and its association with DRMs was enhanced by lysosomal cholesterol loading. Moreover, lysosomal cholesterol deposition was kinetically paralleled by the sequestration of sphingolipids and formation of multilamellar bodies in late endocytic organelles. These results suggest that late endocytic organelles are normally raft-poor and that endocytosed LDL-cholesterol is efficiently recycled to the plasma membrane in an NPC1-dependent process. The cholesterol-sphingolipid accumulation characteristic to NPC disease, and potentially to other sphingolipidoses, causes an overcrowding of rafts forming lamellar bodies in the degradative compartments.

3.184 The *Leishmania* ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase
Legare, D., et al
J. Biol. Chem., ***276(28)****, 26301-26307 (2001)*

The *Leishmania* ATP-binding cassette (ABC) transporter PGPA is involved in metal resistance (arsenicals and antimony), although the exact mechanism by which PGPA confers resistance to antimony, the first line drug against *Leishmania*, is unknown. The results of co-transfection experiments, transport assays, and the use of inhibitors suggest that PGPA recognizes metals conjugated to gluthathione or trypanothione, a glutathione-spermidine conjugate present in *Leishmania*. The HA epitope tag of the influenza hemagglutinin as well as the green fluorescent protein (GFP) were fused at the COOH-terminus of PGPA. Immunofluorescence, confocal and electron microscopy studies of the fully functional tagged molecules clearly indicated that PGPA is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite. Subcellular fractionation of *Leishmania tarentolae* PG-PAHA transfectants was performed to characterize further this ABC transporter. The basal PGPA ATPase activity was determined to be 115 nmoles/mg/min. Transport experiments using radioactive arsenite-glutathione conjugates clearly showed that PGPA recognizes and actively transport thiol-metal conjugates. Overall, the results are consistent with PGPA being an intracellular ABC transporter that confers arsenite and antimonite resistance by sequestration of the metal-thiol conjugates.

3.185 Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole
Kim, J. et al
J. Cell Biol., ***153(2)****, 381-396 (2001)*

Three overlapping pathways mediate the transport of cytoplasmic material to the vacuole in *Saccharomyces cerevisiae*. The cytoplasm to vacuole targeting (Cvt) pathway transports the vacuolar hydrolase, aminopeptidase I (API), whereas pexophagy mediates the delivery of excess peroxisomes for degradation. Both the Cvt and pexophagy pathways are selective processes that specifically recognize their cargo. In contrast, macroautophagy nonselectively transports bulk cytosol to the vacuole for recycling. Most of the import machinery characterized thus far is required for all three modes of transport. However,

unique features of each pathway dictate the requirement for additional components that differentiate these pathways from one another, including at the step of specific cargo selection.

We have identified Cvt9 and its *Pichia pastoris* counterpart Gsa9. In *S. cerevisiae*, Cvt9 is required for the selective delivery of precursor API (prAPI) to the vacuole by the Cvt pathway and the targeted degradation of peroxisomes by pexophagy. In *P. pastoris*, Gsa9 is required for glucose-induced pexophagy. Significantly, neither Cvt9 nor Gsa9 is required for starvation-induced nonselective transport of bulk cytoplasmic cargo by macroautophagy. The deletion of *CVT9* destabilizes the binding of prAPI to the membrane and analysis of a cvt9 temperature-sensitive mutant supports a direct role of Cvt9 in transport vesicle formation. Cvt9 oligomers peripherally associate with a novel, perivacuolar membrane compartment and interact with Apg1, a Ser/Thr kinase essential for both the Cvt pathway and autophagy. In *P. pastoris* Gsa9 is recruited to concentrated regions on the vacuole membrane that contact peroxisomes in the process of being engulfed by pexophagy. These biochemical and morphological results demonstrate that Cvt9 and the *P. pastoris* homologue Gsa9 may function at the step of selective cargo sequestration.

3.186 Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the α-secretase ADAM 10
Kojro, E., Gimpl, G., Lammich, S., Marz, W. and Fahrenholz, F.
Proc. Natl. Acad. Sci., ***98(10),*** *5815-5820 (2001)*

Biochemical, epidemiological, and genetic findings demonstrate a link between cholesterol levels, processing of the amyloid precursor protein (APP), and Alzheimer's disease. In the present report, we identify the α-secretase ADAM 10 (a *d*isintegrin and *m*etalloprotease) as a major target of the cholesterol effects on APP metabolism. Treatment of various peripheral and neutral cell lines with either the cholesterol-extracting agent methyl-β-cyclodextrin or the hydroxymethyl glutaryl-CoA reductase inhibitor lovastin resulted in a drastic increase of secreted α-secretase cleaved soluble APP. This strong stimulatory effect was in the range obtained with phorbol esters and was further increased in cells overexpressing ADAM 10. In cells overexpressing APP, the increase of α-secretase activity resulted in a decreased secretion of Aβ peptides. Several mechanisms were elucidated as being the basis of enhanced α-secretase activity: increased membrane fluidity and impaired internalization of APP were responsible for the effect observed with methyl-β-cyclodextrin; treatment with lovastatin resulted in higher expression of the α-secretase ADAM 10. Our results demonstrate that cholesterol reduction promotes the nonamyloidogenic α-secretase pathway and the formation of neuroprotective α-secretase cleaved soluble APP by several mechanisms and suggest approaches to prevention of or therapy for Alzheimer's disease.

3.187 Neuregulin-1 proteins in rat brain and transfected cells are localized to lipid rafts
Frenzel, K.E. and Falls, D.L.
*J. Neurochem., **77**, 1-12 (2001)*

Neuregulin–1 proteins and their receptors, which are members of the ErbB subfamily of receptor tyrosine kinases, play essential roles in the development of the nervous system and heart. Most neuregulin-1 isoforms are synthesized as transmembrane proproteins that are proteolytically processed to yield an N-terminal fragment containing the bioactive EGF-like domain. In this study we investigated whether neuregulins are found in lipid rafts, membrane microdomains hypothesized to have important roles in signal transduction, protein trafficking, and proteolytic processing. We found that 45% of a 140-kDa neuregulin protein in rat brain synatosomal plasma membrane fractions was insoluble in 1% Triton X-100. Flotation gradient analysis demonstrated the presence of the brain 140-kDa neuregulin protein in low-density fractions enriched in PSD-95, a known lipid raft protein. In transfected cells expressing the neuregulin I-β1a or the III-β1a isoform, most of the neuregulin proprotein was insoluble in 1% Triton X-100, and neuregulin proproteins and C-terminal fragments were detected in lipid raft fractions. In contrast, the III-β1a N-terminal fragment was detected only in the detergent-soluble fraction. These results suggest that localization of neuregulins to lipid rafts may play a role in neuregulin signaling within the nervous system.

3.188 Yeast Rab GTPase-activating protein Gyp1p localizes to the Golgi apparatus and is a negative regulator of Ypt1p
Du, L-L and Novick, P.
*Mol. Biol. Cell, **12**, 1215-1226 (2001)*

A family of related proteins in yeast *Saccharomyces cerevisiae* is known to have in vitro GTPase-activating protein activity on the Rab GTPases. However, their in vivo function remains obscure. One of

them, Gyp1p, acts on Sec4p, Ypt1p, Ypt7p and Ypt51p in vitro. Here, we present data to reveal its in vivo substrate and the role that it plays in the function of the Rab GTPase. Red fluorescent protein-tagged Gyp1p is concentrated on cytoplasmic punctate structures that largely colocalize with a *cis*-Golgi marker. Subcellular fractionation of a yeast lysate confirmed that Gyp1p is peripherally associated with membranes and that it cofractionates with Golgi markers. This localization suggests that Gyp1p may only act on Rab GTPases on the Golgi. A *gyp1Δ* strain displays a growth defect on synthetic medium at 37°C. Overexpression of Ypt1p, but not other Rab GTPases, strongly inhibits the growth of *gyp1*Δ cells. Conversely, a partial loss-of-function allele of *YPT1, ypt1-2*, can suppress the growth defect of *gyp1*Δ cells. Furthermore, deletion of *GYP1* can partially suppress growth defects associated with mutants in subunits of transport protein particle complex, a complex that catalyzes nucleotide exchange on Ypt1p. These results establish that Gyp1p functions on the Golgi as a negative regulator of Ypt1p.

3.189 SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis
Lang, T. et al
EMBO J., ***20(9)***, *2202-2213 (2001)*

During exocytosis, SNARE proteins of secretory vesicles interact with the corresponding SNARE proteins in the plasmalemma to initiate the fusion reaction. However, it is unknown whether SNAREs are uniformly distributed in the membrane or whether specialized fusion sites exist. Here we report that in the plasmalemma, syntaxins are concentrated in 200 nm large, cholesterol-dependent clusters at which secretory vesicles preferentially dock and fuse. The syntaxin clusters are distinct from cholesterol-dependent membrane rafts since they are Triton X-100 soluble and do not co-patch with raft markers. Synaptosomal-associated protein (SNAP)-25 is also clustered in spots, which partially overlap with syntaxin. Cholesterol depletion causes dispersion of these clusters, which is associated with a strong reduction in the rate of secretion, whereas the characteristics of individual exocytic events are unchanged. This suggests that high local concentrations of SNAREs are required for efficient fusion.

3.190 WFS1 (Wolfram syndrome 1) gene product: predominant subcellular localization to endoplasmic reticulum in cultured cells and neuronal expression in rat brain
Takeda, K. et al
Hum. Mol. Genet., ***10(5)***, *477-484 (2001)*

Wolfram (DIDMOAD) syndrome is an autosomal recessive neurodegenerative disorder accompanied by insulin-dependent diabetes mellitus and progressive optic atrophy. Recent positional cloning led to identification of the *WFS1* (Wolfram syndrome 1) gene, a member of a novel gene family of unknown function. In this study, we generated a specific antibody against the C-terminus of the WFS1 protein and investigated its subcellular localization in cultured cells. We also studied its distribution in the rat brain. Biochemical studies indicated the WFS1 protein to be an integral, endoglycosidase H-sensitive membrane glycoprotein that localizes primarily in the endoplasmic reticulum (ER). Consistent with this, immunofluorescence cell staining of overexpressed WFS1 showed a characteristic reticular pattern over the cytoplasm and overlapped with the ER marker staining. No co-localization of WFS1 with mitochondria argues against an earlier clinical hypothesis that Wolfram syndrome is a mitochondria-mediated disorder. In the rat brain, at both the protein and mRNA level, WFS1 was found to be present predominantly in selected neurons in the hippocampus CA1, amygdaloid areas, olfactory tubercle and superficial layer of the allocortex. These expression sites, i.e. components of the limbic system or structures closely associated with this system, may be involved in the psychiatric, behavioral and emotional abnormalities characteristic of this syndrome. ER localization of WFS1 suggests that this protein plays an as yet undefined role in membrane trafficking, protein processing and/or regulation of ER calcium homeostasis. These studies represent a first step toward the characterization of WFS1 protein, which presumably functions to maintain certain populations of neuronal and endocrine cells.

3.191 Regulation of tumor angiogenesis by oxygen-regulated protein 150, an inducible endoplasmic reticulum chaperone
Ozawa, K. et al
Cancer Res., ***61***, *4206-4213 (2001)*

Expression of angiogenic factors such as vascular endothelial growth factor (VEGF) under conditions of cell stress involves both transcriptional and translational events, as well as an important role for inducible endoplasmic reticulum (ER) chaperones. Coexpression of VEGF and 150-kDa oxygen-regulated protein

(ORP), a novel ER chaperone, in human glioblastoma suggested a link between angiogenesis and ORP150. C6 glioma cells stably transfected with ORP150 antisense displayed selectively reduced ORP150 expression. Tumors raised after inoculation of immuno-compromised mice with ORP150 antisense C6 glioma transfect-ants demonstrated an initial phase of growth comparable to wild-type C6 glioma cells which was followed by marked regression within 8 days. Decreased density of platelet/endothelial cell adhesion molecule 1-positive structures within the tumor bed was consistent with reduced angiogenesis in C6 gliomas expressing ORP150 antisense, compared with tumors derived from C6 cells overexpressing ORP150 sense or vector controls. *In vitro*, inhibition of ORP150 expression decreased release of VEGF into culture supernatants; in ORP150 antisense transfectants, VEGF accumulated intracellularly within the ER. These findings demons-trate a critical role for the inducible ER chaperone ORP150 in tumor-mediated angiogenesis via processing of VEGF, and thus, highlight a new facet of mechanism amenable to therapeutic manipulation in tumors.

3.192 Subcellular compartment and molecular subdomain of β-amyloid precursor protein relevant to the Aβ42-promoting effects of Alzheimer mutant presenilin 2
Iwata, H., Tomita, T., Maruyama, H. And Iwatsubo, T.
J. Biol. Chem., ***276(24)***, *21678-21685 (2001)*

Increased production of amyloidβ peptides ending at position 42 (Aβ42) is one of the pathogenic phenotypes caused by mutant forms of presenilins (PS) linked to familial Alzheimer's disease. To identify the subcellular compartment(s) in which familial Alzheimer's disease mutant PS2 (mt PS2) affects the γ-cleavage of βAPP to increase Aβ42, we co-expressed the C-terminal 99-amino acid fragment of βAPP (C100) tagged with sorting signals to the endoplasmic reticulum (C100/ER) or to the *trans*-Golgi network (C100/TGN) together with mt PS2 in N2a cells. C100/TGN co-transfected with mt PS2 increased levels or ratios of intracellular as well as secreted Aβ42 at similar levels to those with C100 without signals (C100/WT), whereas C100/ER yielded a negligible level of Aβ, which was not affected by co-transfection of mt PS2. To identify the molecular subdomain of βAPP required for the effects of mt PS2, we next co-expressed C100 variously truncated at the C-terminal cytoplasmic domain together with mt PS2. All types of C-terminally truncated C100 variants including that lacking the entire cytoplasmic domain yielded the secreted form of Aβ at levels comparable with those from C100/WT, and co-transfection of mt PS2 increased the secretion of Aβ42. These results suggest that (i) late intracellular compartments including TGN are the major sites in which Aβ42 is produced and up-regulated by mt PS2 and that (ii) the anterior half of C100 lacking the entire cytoplasmic domain is sufficient for the overproduction of Aβ42 caused by mt PS2.

3.193 *Toxoplasma* evacuoles: a two-step process of secretion and fusion forms the parasitophorous vacuole
Hakansson, S., Charron, A.J. and Sibley, L.D.
The EMBO J., ***20(12)***, *3132-3144 (2001)*

Rapid discharge of secretory organelles called rhoptries is tightly coupled with host cell entry by the protozoan parasite *Toxoplasma gondii*. Rhoptry contents were deposited in clusters of vesicles within the host cell cytosol and within the parasitophorous vacuole. To examine the fate of these rhoptry-derived secretory vesicles, we utilized cytohalasin D to prevent invasion, leading to accumulation of protein-rich vesicles in the host cell cytosol. These vesicles lack an internal parasite and are hence termed evacuoles. Like the mature parasite-containing vacuole, evacuoles became intimately associated with host cell mitochondria and endoplasmic reticulum, while remaining completely resistant to fusion with host cell endo-somes and lysosomes. In contrast, evacuoles were recruited to pre-existing, parasite-containing vacuoles and were capable of fusing and delivering their contents to these compartments. Our findings indicate that a two-step process involving direct rhoptry secretion into the host cell cytoplasm followed by incorporation into the vacuole generates the parasitophorous vacuole occupied by *Toxoplasma*. The characteristic properties of the mature vacuole are likely to be determined by this early delivery of rhoptry components.

3.194 Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation and environmental stress in *Trypanosoma cruzi*
Ruiz, F.A., Rodrigues, C.O. and Docampo, R.
J. Biol. Chem., ***276(28)***, *26114-26121 (2001)*

Inorganic polyphosphate (polyP) has been identified and measured in different stages of *Trypanosoma cruzi*. Millimolar levels (in terms of P_i residues) in chains of less than 50 residues long, and micromolar levels in chains of about 700-800 residues long, were found in different stages of *T. cruzi*. Analysis of purified *T. cruzi* acidocalcisomes indicated that polyPs were preferentially located in these organelles. This was confirmed by visualization of polyPs in the acidocalcisomes using 4,6-diamidino-2-phenylindole (DAPI). A rapid increase (within 2-4 h) in the levels of short and long-chain polyPs was detected during trypomastigote to amastigote differentiation and during the lag phase of growth of epimastigotes (within 12-24 h). Levels rapidly decreased after epimastigotes resumed growth. Short– and long-chain polyP levels rapidly decreased upon exposure of epimastigotes to hypo-osmotic or alkaline stresses while levels increased after hyperosmotic stress. Ca^{2+} release from acidocalcisomes by a combination of ionophores (ionomycin, nigericin) was associated with the hydrolysis of short – and long-chain polyPs. In agreement with these results, acidocalcisomes were shown to contain polyphosphate kinase and exopolyphosphatase activities. Together, these results suggest a critical role for these organelles in the adaptation of the parasite to environmental changes.

3.195 Apg2 is a novel protein required for the cytoplasm to vacuole targeting, autophagy, and pexophagy pathways

Wang, C-W., et al
J. Biol. Chem, ***276(32)****, 30442-30451 (2001)*

To survive starvation conditions, eukaryotes have developed an evolutionarily conserved process, termed autophagy, by which the vacuole/lysosome mediates the turnover and recycling of non-essential intracellular material for re-use in critical biosynthetic reactions. Morphological and biochemical studies in *Saccharomyces cerevisiae* have elucidated the basic steps and mechanisms of the autophagy pathway. Although it is a degradative process, autophagy shows substantial overlap with the biosynthetic cytoplasm to vacuole targeting (Cvt) pathway that delivers resident hydrolases to the vacuole. Recent molecular genetics analyses of mutants defective in autophagy and the Cvt pathway, *apg*, *aut* and *cvt*, have begun to identify the protein machinery and provide a molecular resolution of the sequestration and import mechanism that are characteristic of these pathways. In this study, we have identified a novel protein, termed Apg2, required for both the Cvt and autophagy pathways as well as the specific degradation of peroxisomes. Apg2 is required for the formation and/or completion of cytosolic sequestering vesicles that are required for vacuolar import through both the Cvt pathway and autophagy. Biochemical studies revealed that Apg2 is a peripheral membrane protein. Apg2 localizes to the previously identified perivacuolar compartment that contains Apg9, the only characterized integral membrane protein that is required for autophagosome/Cvt vesicle formation.

3.196 Apg2p functions in autophagosome formation on the perivacuolar structure

Shintani, T., Suzuki, K., Kamada, Y., Noda, T. and Ohsumi,Y.
J. Biol. Chem., ***276(32)****, 30452-30460 (2001)*

Autophagy is a degradative process in which cytoplasmic components are non-selectively sequestered by double-membrane structures, termed autophagosomes, and transported to the vacuole. We have identified and characterized a novel protein Apg2 essential for autophagy in yeast. Biochemical and fluorescence microscopic analyses indicate that Apg2p functions at the step of autophagosome formation. Apg2p localizes to some membranous structure distinct from any known organelle. Using fluorescent protein-tagged Apg2p, we showed that Apg2p localizes to a dot structure close to the vacuole, where Apg8p also exists, but not on autophagosomes unlike Apg8p. This punctate localization of Apg2p depends on the function of Apg1p kinase, phosphatidylinositol-3 kinase complex and Apg9p. $Apg2p^{G83E}$, encoded by an *apg2-2* allele, shows a severely reduced activity of autophagy and a dispersed localization in the cytoplasm. Overexpression of the mutant Apg2p lessens the defect in autophagy. These results suggest that the dot structure is physiologically important. Apg2p and Apg8p are independently recruited to the structure but coordinately function there to form the autophagosome.

3.197 Cross-talk between caveolae and glycosylphosphatidylinositol-rich domains

Abrami, L., et al.
J. Biol. Chem., ***276(33)****, 30729-30736 (2001)*

Most mammalian cells have in their plasma membrane at least two types of lipid microdomains, non-invaginated lipid rafts and caveolae. Glycosylphosphatidylinositol (GPI)-anchored proteins constitute a class of proteins that are enriched in rafts but not caveolae at steady state. We have analyzed what the

effects of abolishing GPI-biosynthesis are on rafts, caveolae and cholesterol levels. GPI-deficient cells were obtained by screening for resistance to the pore-forming toxin aerolysin, which uses this class of proteins as receptors. Despite the absence of GPI-anchored proteins, mutant cells still contained lipid rafts indicating that GPI-anchored proteins are not crucial structural elements of these domains. Interestingly, the caveolae-specific membrane proteins, caveolin 1 and 2, were up regulated in GPI-deficient cells, in contrast to flotillin-1 and GM1, which were expressed at normal levels. Also the number of surface caveolae was increased. This effect was specific since recovery of GPI biosynthesis by gene recomplementation, restored caveolin expression and the number of surface caveolae to wild type levels. The inverse correlation between the expression of GPI-anchored proteins and caveolin-1 was confirmed by the observation that overexpression of caveolin-1 in wild type cells led to a decrease in the expression of GPI-anchored proteins. In cells lacking caveolae, the absence of GPI-anchored proteins caused an increase in cholesterol levels suggesting a possible role of GPI-anchored proteins in cholesterol homeostasis, which in some cells, such as Chinese hamster ovary cells, can be compensated by caveolin up regulation.

3.198 Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction
Liang, X et al
J. Biol. Chem., ***276(33)****, 30987-30994 (2001)*

Fatty acylation of Src family kinases is essential for localization of the modified proteins to the plasma membrane and to plasma membrane rafts. It has been suggested that the presence of saturated fatty acyl chains on proteins is conducive for their insertion into the liquid ordered lipid domains present in rafts. The ability of unsaturated dietary fatty acids to be attached to Src family kinases has not been investigated. Here, we demonstrate that heterogeneous fatty acylation of Src family kinases occurs and that the nature of the attached fatty acid influences raft-mediated signal transduction. By using matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry, we show that in addition to 14:0 (myristate), 14:1 and 14:2 fatty acids can be attached to the N-terminal glycine of the Src family kinase Fyn when the growth media is supplemented with these dietary fatty acids. Moreover, we synthesized novel iodinated analogs of oleate and stearate, and showed that heterogeneous S-acylation can occur on cysteine residues within Fyn as well as Gα, GAP43 and Ras. Modification of Fyn with unsaturated or polyunsaturated fatty acids reduced its raft localization and resulted in decreased T cell signal transduction. These studies establish that heterogeneous fatty acylation is a widespread occurrence that serves to regulate signal transduction by membrane bound proteins.

3.199 Lipoprotein lipase and leptin are accumulated in different secretory compartments in rat adipocytes
Roh, C., Roduit, R., Thorens, B., Fried, S. and Kandror, K.V.
J. Biol. Chem., ***276(38)****, 35990-35994 (2001)*

Adipose cells produce and secrete several physiologically proteins, such as lipoprotein lipase (LPL), leptin, adipsin, Acrp30, etc. However, secretory pathways in adipocytes have not been characterized, and vesicular carriers responsible for accumulation and transport of secreted proteins have not been identified. We have compared the intracellular localization of two proteins secreted from adipose cells: leptin and LPL. Adipocytes accumulate large amounts of both proteins, suggesting that neither of them is targeted to the constitutive secretory pathway. By means of velocity centrifugation in sucrose gradients, equilibrium density centrifugation in **iodixanol** gradients and immunofluorescence confocal microscopy, we determined that LPL and leptin were localized in different membrane structures. LPL was found mainly in the endoplasmic reticulum with a small pool being present in low-density membrane vesicles that may represent a secretory compartment in adipose cells. Virtually all intracellular leptin was localized in these low-density secretory vesicles. Insulin-sensitive Glut4-vesicles did not contain either LPL or leptin. Thus, secretion from adipose cells is controlled both at the exit from the endoplasmic reticulum as well as at the level of "downstream" secretory vesicles.

3.200 Activation of mitogen-activated protein kinase by membrane targeted Raf chimeras is independent of raft localization
Chen, X. and Resh, M.D.
J. Biol. Chem., ***276(37)****, 34617-34623 (2001)*

Binding of proteins to the plasma membrane can be achieved with various membrane targeting motifs, including combinations of fatty acids, isoprenoids, and basic domains. In this study, we investigate whether attachment of different membrane targeting motifs influences the signaling capacity of membrane-

bound signal transduction proteins by directing the proteins to different membrane microdomains. We used c-Raf-1 as a model for a signaling protein that is activated when membrane-bound. Three different membrane targeting motifs from K-Ras, Fyn and Src proteins were fused to the N- or C-terminus of Raf-1. The ability of the modified Rafs to initiate MAPK signaling was then investigated. All three modified Raf-1 constructs activated MAPK to nearly equivalent levels. The extent of localization of the Raf-1 constructs to membrane microdomains known as rafts did not correlate with the level of MAPK activation. Moreover, treatment of cells with the raft disrupting drug methyl-β-cyclodextrin (MβCD) caused activation of MAPK to levels equivalent to those achieved with membrane targeted Raf constructs. The use of pharmacological agents as well as dominant mutants revealed that MAPK activation by MβCD proceeds via a PI3K dependent mechanism that is Ras/Raf independent. We conclude that cholesterol depletion from the plasma membrane by MβCD constitutes an alternative pathway for activating MAPK.

3.201 Biochemical and morphological analysis on the localization of Rac1 in neurons
Kumanogoh, H., Miyata, S., Sokawa, Y. and Maekawa, S.
*Neuroscience Res., **39**, 189-196 (2001)*

The acquisition of cell type-specific morphologies is a central feature of neuronal differentiation. Many extra- and intracellular signals are known to cause the morphological changes of neuronal cells through the reconstruction of the microfilaments underneath the cell membrane. The membrane microdomain called "raft" has been paid much attention, for this domain contains many signal-transducing molecules including trimeric G proteins and cytoskeletal proteins. The raft domain is recovered in a low-density fraction after the treatment of the membrane with the non-ionic detergent such as Triton X-100 and the enrichment of cholesterol and sphingolipids is ascribed to be responsible for the detergent insolubility. In contrast to the well-known localization of trimeric G proteins in raft, the localization of small G proteins in the raft is poorly characterized. Since Rho family small G proteins (Rho, Rac, and Cdc42) regulate the microfilament system, we studied the localization of Rho family small G proteins in the raft of rat brain with western blotting. Specific localization of Rac1 was detected in the raft from 1-day-old and 8-week-old rat whole brain, and also in the raft prepared from the growth cone and synaptic plasma membrane fractions. Rho and Cdc42 were, in contrast, recovered in the Triton soluble fraction. Double immunostaining of cultured hippocampal neurons with antibodies to Rac1 and MAP-2, or Rac1 and tau, showed punctate distribution of Rac1 in axons as well as in dendrites.

3.202 Serum-activated assembly and membrane translocation of an endogenous Rac1: effector complex
Hansen, M.D.H. and Nelson, W.J.
*Current Biology, **11**, 356-360 (2001)*

Rho family GTPases (Cdc42, Rac1, and RhoA) function downstream of Ras, and in a variety of cellular processes. Studies to examine these functions have not directly linked endogenous protein interactions with specific in vivo functions of Rho GTPases. Here, we show that endogenous Rac1 and two known binding partners, Rho GDP dissociation inhibitor (RhoGDI) and p21-activated kinase (PAK), fractionate as distinct cytosolic complexes. A Rac1: PAK complex is translocated from the cytosol to ruffling membranes upon cell activation by serum. Overexpression of dominant-negative (T17N) Rac1 does not affect the assembly or distribution of this Rac1: PAK complex. This is the first direct evidence of how a specific function of Rac1 is selected by the assembly and membrane translocation of a distinct Rac1:effector complex.

3.203 Subcellular study of sphingoid base phosphorylation in rat tissues: evidence for multiple sphingosine kinases
Gijsbers, S., Van der Hoeven, G. And Van Veldhoven, P.P.
*Biochim. Biophys. Acta, **1532**, 37-50 (2001)*

The enzymatic phosphorylation of sphingoid bases was analysed in rat tissues, using D-*erythro*-[4,5-^{3}H]sphinganine as substrate. After optimization of the assay, taking care to block sphingosine-phosphate lyase and sphingosine phosphatase, highest ATP-dependent kinase activities were present in testis, followed by kidney, and intestinal mucosa. Approximately two thirds of the kidney activity was membrane bound, the remaining being cytosolic. Classical cell fractionation studies of a particulate fraction from kidney homogenates by Percoll gradient and sucrose density gradient centrifugation revealed that kinase activities are associated with vesicles derived from the endoplasmic reticulum and the plasma membrane. Based on indirect data, such as the effect of detergents and divalent ions, the cytosolic and both membrane

bound activities appear to reside in different proteins. *N,N*-dimethylsphingenine was inhibitory to all three different kinases, which were mainly active towards the D-erythro isomers of sphingenine and sphinganine.

3.204 GPI-anchored proteins and glycoconjugates segregate into lipid rafts in Kinetoplastida
Denny, P.W., Field, M.C. and Smith, D.F.
FEBS Lett., ***491***, *148-153 (2001)*

The plasma membranes of the divergent eukaryotic parasites, *Leishmania* and *Trypanosoma*, are highly specialized, with a thick coat of glycoconjugates and glycoproteins playing a control role in virulence. Usually, the majority of these surface macromolecules are attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. In mammalian cells and yeast, many GPI-anchored molecules associate with sphingolipid and cholesterol-rich detergent-resistant membranes, known as lipid rafts. Here we show that GPI-anchored parasite macromolecules (but not the dual acylated *Leishmania* surface protein (hydrophilic acylated surface protein) or a subset of the GPI-anchored glycoinositol phopholipid glycolipids) are enriched in a sphingolipid/sterol-rich fraction resistant to cold detergent extraction. This observation is consistant with the presence of functional lipid rafts in these ancient, highly polarized organisms.

3.205 Endoplasmic reticulum and *cis*-Golgi localization of human T-lymphotropic virus type 1 $p12^I$: association with calreticulin and calnexin
Ding, W. et al
J. Virol., ***75(16)***, *7672-7682 (2001)*

Human T-lymphotropic virus type 1 (HTLV-1) is a complex retrovirus encoding regulatory and accessory genes in four open reading frames (ORF I to IV) of the pX region. We have demonstrated an important role of pX ORF I expression, which encodes $p12^I$, in establishment of HTLV-1 in a rabbit model and for optimal viral infectivity in quiescent primary lymphocytes. These data indicated that $p12^I$ may enhance lymphocyte activation and thereby promote virus infection. To further define the role of $p12^I$ in cell activation, we characterized the subcellular localization of $p12^I$ transfected 293T cells and HeLa-Tat cells by multiple methods, including immunofluorescence confocal microscopy, and subcellular fractionation. Herein, we demonstrate that $p12^I$ accumulates in the endoplasmic reticulum (ER) and *cis*-Golgi apparatus. The localization of $p12^I$ was unchanged following treatments with both cycloheximide (blocking de novo protein synthesis) and brefeldin A (disrupting ER-to-Golgi protein transport), indicating that the protein is retained in the ER and *cis*-Golgi. Moreover, using coimmunoprecipitation assays, we identify the direct binding of $p12^I$ with both calreticulin and calnexin, resident ER proteins which regulate calcium storage. Our results indicate that $p12^I$ directly binds key regulatory proteins involved in calcium-mediated cell signalling and suggest a role of $p12^I$ in the establishment of HTLV-1 by activation of host cells.

3.206 Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains
Lindwasser, O.W. and Resh, M.D.
J. Virol., ***75(17)***, *7913-7924 (2001)*

The Gag polyprotein of human immunodeficiency virus type 1 (HIV-1) organizes the assembly of nascent virions at the plasma membrane of infected cells. Here we demonstrate that a population of Gag is present in distinct raft-like membrane microdomains that we have termed “barges”. Barges have a higher density than standard rafts, most likely due to the presence of oligomeric Gag-Gag assembly complexes. The regions of the Gag protein responsible for barge targeting were mapped by examining the flotation behavior of wild-type and mutant proteins on **OptiPrep** density gradients. N-myristoylation of Gag was necessary for association with barges. Removal of the NC and p6 domains shifted much of the Gag from barges into typical raft fractions. These data are consistent with a model in which multimerization of myristoylated Gag proteins drive association of Gag oligomers into raft-like barges. The functional significance of barge association was revealed by several lines of evidence. First, Gag isolated from virus-like particles was almost entirely localized in barges. Moreover, a comparison of wild-type Gag with Fyn(10)Gag, a chimeric protein containing the N-terminal sequence of Fyn, revealed that Fyn(10)Gag exhibited increased affinity for barges and a two- to fourfold increase in particle production. These results imply that association of Gag with raft-like barge membrane microdomains plays an important role in the HIV-1 assembly process.

3.207 Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization

Gomez-Mouton, C. et al

*Proc. Natl. Acad, Sci., USA, **98(17)**, 9642-9647 (2001)*

Redistribution of specialized molecules in migrating cells develops asymmetry between two opposite cell poles, the leading edge and the uropod. We show that acquisition of a motile phenotype in T lymphocytes results in the asymmetric redistribution of ganglioside GM3- and GM1-enriched raft domains to the leading edge and to the uropod, respectively. This segregation to each cell pole parallels the specific redistribution of membrane proteins associated to each raft subfraction. Our data suggest that raft partitioning is a major determinant for protein redistribution in polarized T cells, as ectopic expression of raft-associated proteins results in their asymmetric redistribution, whereas non-raft partitioned mutants of these proteins are distributed homogeneously in the polarized cell membrane. Both acquisition of a migratory phenotype and SDF-1 α-induced chemotaxis are cholesterol depletion-sensitive. Finally, GM3 and GM1 raft redistribution requires an intact actin cytoskeleton, but is insensitive to microtubule disruption. We propose that membrane protein segregation not only between raft and nonraft domains but also between distinct raft subdomains may be an organizational principle that mediates redistribution of specialized molecules needed for T cell migration.

3.208 The Sec6/8 complex in mammalian cells: characterization of mammalian Sec3, subunit interactions, and expression of subunits in polarized cells

Matern, H.T., Yeaman, C., Nelson, W.J. and Scheller, R.H.

*Proc. Natl. Acad. Sci, USA, **98(17)**, 9648-9653 (2001)*

The yeast exocyst complex (also called Sec6/8 complex in higher eukaryotes) is a multiprotein complex essential for targeting exocytic vesicles to specific docking sites on the plasma membrane. It is composed of eight proteins (Sec3, -5, -6, -8, -10, and -15, and Exo70 and -84), with molecular weights ranging from 70 to 144 kDa. Mammalian orthologues for seven of these proteins have been described and here we report the cloning and initial characterization of the remaining subunit, Sec3. Human Sec3 (hSec3) shares 17% sequence identity with yeast Sec3p, interacts in the two-hybrid system with other subunits of the complex (Sec5 and Sec8), and is expressed in almost all tissues tested. In yeast, Sec3p has been proposed to be a spatial landmark for polarized secretion (1), and its localization depends on its interaction with Rho1p (2). We demonstrate here that hSec3 lacks the potential Rho1-binding site and GFP-fusions of hSec3 are cytosolic. Green fluorescent protein (GFP)-fusion of nearly every subunit of the mammalian Sec6/8 complex were expressed in Madin-Darby canine kidney (MDCK) cells, but they failed to assemble into a complex with endogenous proteins and localized in the cytosol. Of the subunits tested, only GFP-Exo70 localized to lateral membrane sites of cell-cell contact when expressed in MDCK cells. Cells overexpressing GFP-Exo70 fail to form a tight monolayer, suggesting the Exo70 targeting interaction is critical for normal development of polarized epithelial cells.

3.209 Nerve growth factor activates persistent Rap1 signaling in endosomes

Wu, C., Lai, C-F. and Mobley, W.C.

*J. Neurosci., **21(15)**, 5406-5416 (2001)*

We investigated a role for endogenous Rap1, a small monomeric GTP-binding protein of the Ras family, in nerve growth factor (NGF) signaling in PC12 cells. Although both epidermal growth factor (EGF) and NGF caused transient activation of Ras, only NGF induced the activation of Rap1. Moreover, Rap1 activation was sustained for hours, an effect that matched the sustained activation of the mitogen-activated protein kinase (MAPK) pathway. To investigate the molecular basis for Rap1 activation, we examined complexes containing C3G, a guanine nucleotide exchange factor for Rap1, and CrkL, an adapter protein known to influence Rap1 signaling. NGF induced the formation of a long-lived complex containing C3G/CrkL/Shp2/Gab2/TrkA. Linking the complex to Rap1 activation, we coprecipitated activated TrkA and activated MAPK with activated Rap1 in NGF-treated cells. Confocal microscopy and subcellular fractionation showed that activated Rap1 and the other proteins of the signaling complex were present in endosomes. Pretreatment of PC12 cells with brefeldin A (BFA), which disrupts the Golgi and endosomal compartments, had little effect on Ras activation but strongly inhibited NGF-induced Rap1 activation and continuing MAPK activation. We propose that endosomes are a site from which NGF induces the prolonged activation of Rap1 and MAPK.

3.210 The first proline of PALP motif at the C terminus of presenilins is obligatory for stabilization, complex formation, and γ-secretase activities of presenilins
Tomita, T. et al
J. Biol. Chem., ***276(35),*** *33273-33281 (2001)*

Mutations in presenilin (PS) genes cause early-onset familial Alzheimer's disease by increasing production of the amyloidogenic form of amyloid β peptides ending at residue 42 (Aβ42). PS is an evolutionarily conserved multipass transmembrane protein, and all known PS proteins contain a proline-alanine-leucine-proline (PALP) motif starting at proline (P) 414 (amino acid numbering based on human PS2) at the C terminus. Furthermore, missense mutations that replace the first proline of PALP with leucine (P414L) lead to a loss-of-function of PS in *Drosophila melanogaster* and *Caenorhabditis elegans*. To elucidate the roles of the PALP motif in PS structure and function, we analyzed neuro2a as well as PS1/2 null fibroblast cell lines transfected with human PS harboring mutations at the PALP motif. P414L mutation in PS2 (and its equivalent in PS1) abrogated stabilization, high molecular weight complex formation, and entry to Golgi/*trans*-Golgi network of PS proteins, resulting in failure of Aβ42 overproduction on familial Alzheimer's disease mutant basis as well as of site-3 cleavage of Notch. These data suggest that the first proline of the PALP motif plays a crucial role in the stabilization and formation of the high molecular weight complex of PS, the latter being the active form with intramembrane proteolytic activities.

3.211 A role for smooth endoplasmic reticulum membrane cholesterol ester in determining the intracellular location and regulation of sterol-regulatory element-binding protein-2
Iddon, R. et al
Biochem. J., ***358****, 415-422 (2001)*

Cellular cholesterol homoeostasis is regulated through proteolysis of the membrane-bound precursor sterol-regulatory element-binding protein (SREBP) that releases the mature transcription factor form, which regulates gene expression. Our aim was to identify the nature and intracellular site of the putative sterol-regulatory pool which regulates SREBP proteolysis in hamster liver. Cholesterol metabolism was modulated by feeding hamsters control chow, or a cholesterol-enriched diet, or by treatment with simvastatin or with the oral acyl-CoA: cholesterol acyltransferase inhibitor C1-1011 plus cholesterol. The effects of the different treatments on SREBP activation were confirmed by determination of the mRNAs for the low-density lipoprotein receptor and hydroxymethylglutaryl-CoA (HMG-CoA) reductase and by measurement of HMG-CoA reductase activity. The endoplasmic reticulum was isolated from livers and separated into subfractions by centrifugation in self-generating iodixanol gradients. Immunodetectable SREBP-2 accumulated in the smooth endoplasmic reticulum of cholesterol-fed animals. Cholesterol ester levels of the smooth ER membrane (but not the cholesterol levels) increased after cholesterol feeding and fell after treatment with simvastatin or C1-1011. The results suggest that an increased cellular cholesterol load causes accumulation of SREBP-2 in the smooth endoplasmic reticulum and, therefore, that membrane cholesterol ester may be one signal allowing exit of the SREBP-2/SREBP-cleavage-regulating protein complex to the Golgi.

3.212 Intracellular distribution of lysosomal sialidase is controlled by the internalisation signal in its cytoplasmic tail
Lukong, K.E. et al
J. Biol. Chem., ***276(49)****, 46172-46181 (2001)*

Sialidase (neuraminidase), encoded by the *neu-1* gene in the major histocompatibility complex locus catalyzes the intralysosomal degradation of sialylated glycoconjugates. Inherited deficiency of sialidase results in sialidosis or galactosialidosis, both severe metabolic disorders associated with lysosomal storage of oligosaccharides and glycopeptides. Sialidase also plays an important role in cellular signaling and is specifically required for the production of cytokine interleukin-4 by activated T lymphocytes. In these cells, *neu-1*-encoded sialidase activity is increased on the cell surface, suggesting that a specific mechanism regulates sorting of this enzyme to the plasma membrane. We investigated that mechanism by first showing that sialidase contains the internalization signal found in lysosomal membrane proteins targeted to endosomes via clathrin-coated pits. The signal consists of a C-terminal tetrapeptide ^{412}YGTL^{415}, with Tyr^{412} and Leu^{415} essential for endocytosis of the enzyme. We further demonstrated that redistribution of sialidase from lysosomes to the cell surface of activated lymphocytes is accompanied by increased reactivity of the enzyme with antiphosphotyrosine antibodies. We speculate that phosphorylation of Tyr^{412} results in inhibition of sialidase internalization in activated lymphocytes.

3.213 Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm

Simons, M. et al

Am. J. Pathol., ***159(3)****, 1069-1077 (2001)*

NPHS1 has recently been identified as the gene whose mutations cause congenital nephrotic syndrome of the Finnish type. The respective gene product nephrin is a transmembrane protein expressed in glomerular podocytes and primarily localized to the glomerular slit diaphragm. This interpodocyte junction functions in the glomerular filtration by restricting the passage of plasma proteins into the urinary space in a size-selective manner. The functional role of nephrin in this filtration process is so far not very well understood. In this study, we show that nephrin associates in an oligomerized form with signaling microdomains, also known as lipid rafts, and that these localize to the slit diaphragm. We also show that the nephrin-containing rafts can be immunoisolated with the 27A antibody recognizing a podocyte-specific 9-O-acetylated GD3 ganglioside. In a previous study it has been shown that the *in vivo* injection of this antibody leads to morphological changes of the filtration slits resembling foot process effacement. Here, we report that, in this model of foot process effacement, nephrin dislocates to the apical pole of the narrowed filtration slits and also that it is tyrosine phosphorylated. We suggest that lipid rafts are important in the spatial organization of the glomerular slit diaphragm under physiological and pathological conditions.

3.214 Presenilin, notch, and the genesis and treatment of Alzheimer's disease

Selkoe, D.J.

Proc. Natl. Acad, Sci., ***98(20)****, 111039-11041 (2001)*

Elucidation of the proteolytic processing of the amyloid β-protein precursor (APP) has revealed that one of the two proteases (γ-secretase) that cleave APP to release amyloid-β protein (Aβ) is likely to be presenilin. Presenilin also mediates the γ-secretase-like cleavage of Notch receptors to enable signaling by their cytoplasmic domains. Therefore, APP and Notch may be the first identified substrates of a unique intramembranous aspartyl protease that has presenilin as its active-site component. In view of the evidence for a central role of cerebral build-up of Aβ in the pathogenesis of Alzheimer's disease, this disorder appears to have risen in the human population as a late-life consequence of the conservation of a critical developmental pathway.

3.215 Cellular membrane-binding ability of the C-terminal cytoplasmic domain of human immunodeficiency virus type 1 envelope transmembrane protein gp41

Chen, S.S-L., Lee, S-F. and Wang, C-T.

J. Virol., ***75(20)****, 9925-9938 (2001)*

The amphipathic α-helices located in the cytoplasmic tail of the envelope (Env) transmembrane glycoprotein gp41 of human immunodeficiency virus type 1 have been implicated in membrane association and cytopathicity. Deletion of the last 12 amino acids in the C terminus of this domain severely impairs infectivity. However, the nature of the involvement of the cytoplasmic tail in Env-membrane interactions in cells and the molecular basis for the defect in infectivity of this mutant virus are still poorly understood. In this study we examined the interaction of the cytoplasmic tail with membranes in living mammalian cells by expressing a recombinant cytoplasmic tail fragment and an *Escherichia coli* β-galactosidase/cytoplasmic tail fusion protein, both of them lacking gp120, the gp41 ectodomain, and the transmembrane region. We found through cell fractionation, in vivo membrane flotation, and confocal immunofluorescence studies that the cytoplasmic tail contained determinants to be routed to a perinuclear membrane region in cells. Further mapping showed that each of the three lentivirus lytic peptide (LLP-1, LLP-2, and LLP-3) sequences conferred this cellular membrane-targeting ability. Deletion of the last 12 amino acids from the C terminus abolished the ability of the LLP-1 motif to bind to membranes. High salt extraction, in vitro transcription and translation, and posttranslational membrane binding analyses indicated that the β-galactosidase/LLP fusion proteins were inserted into membranes via the LLP sequences. Subcellular fractionation and confocal microscopy studies revealed that each of the LLP motifs, acting in a position-independent manner, targeted non-endoplasmic reticulum (ER)-associated-β-galactosidase and enhanced green fluorescence protein to the ER. Our study provides a basis for the involvement of the gp41 cytoplasmic tail during Env maturation and also supports the notion that the membrane apposition of the C-terminal cytoplasmic tail plays a crucial role in virus-host interaction.

3.216 The polyphosphate bodies of *Chlamydomas reinhardtii* possess a proton pumping pyrophosphatase and are similar to acidocalcisomes

Ruiz, F.A., Marchesini, N., Seufferheld, M., Govindjee and Docampo, R.
J. Biol. Chem., ***276/49***, *46196-46203 (2001)*

Acidocalcisomes are acidic calcium storage compartments described initially in trypanosomatid and apicomplexan parasites. In this work, we describe organelles with properties similar to acidocalcisomes in the green alga *Chlamydomonas reinhardtii*. Nigericin and NH_4CL released $^{45}Ca^{2+}$ from preloaded permeabilized cells suggesting the incorporation of a significant amount of this cation into an acidic compartment. X-ray microanalysis of the electron-dense vacuoles or polyphosphate bodies of *C. reinhardtii* showed large amounts of phosphorus, magnesium, calcium, and zinc. Immunofluorescence microscopy, using antisera raised against a peptide sequence of the vacuolar-type proton pyrophosphatase (H^+-PPase) of *Arabidopsis thaliana* which is conserved in the *C. reinhardtii* enzyme, indicated localization in the plasma membrane, in intracellular vacuoles and the contractile vacuole where it co-localized with the vacuolar proton ATPase (V-H^+-ATPase). Purification of the electron-dense vacuoles using **iodixanol** density gradients indicated a preferential localization of the H^+-PPase and the V-H^+-ATPase activities in addition to high concentrations of PP_i and short and long chain polyphosphate, but lack of markers for mitochondria and chloroplasts. In isolated electron-dense vacuoles, PP_i-driven proton translocation was stimulated by potassium ions and inhibited by the PP_i analog aminomethylene-diphosphonate. Potassium fluoride, imidodiphosphate, *N,N'*-decyclohexylcarbodi-imide and *N*-ethylmaleimide also inhibited PP_i hydrolysis in the isolated organelles in a dose-dependent manner. These results indicate that the electron-dense vacuoles of *C.reinhardtii* are very similar to acidocalcisomes with regard to their chemical composition and the presence of proton pumps. Polyphosphate was also localized to the contractile vacuole by 4', 6-diamino-2-phenylindole staining, suggesting, with the immunochemical data, a link between these organelles and the acidocalcisomes.

3.217 Characterization of a presenilin-mediated APP carboxyl terminal fragment γ: Evidence for distinct mechanisms involved in "gamma-secretase processing of the APP and notch 1 transmembrane domains

Yu, C et al
J. Biol. Chem.,***276(47)***, *43756-43760 (2001)*

A variety of investigations have led to the conclusion that presenilins (PS) play a critical role in intramembranous, γ-secretase proteolysis of selected type I membrane proteins, including Notch1 and amyloid precursoe protein (APP). We now show that the generation of the S3/Notch intracellular domain and APP-carboxy-terminal fragment γ (CTFγ) derivatives are dependent on PS expression and inhibited by a highly selective and potent γ-secretase inhibitor. Unexpectedly, the APP-CTFγ derivative is generated by processing between Leu645 and Val646 (of APP_{695}), several amino acids carboxyl-terminal to the scissile bonds for production of amyloid β protein peptides. Although the relationship of APP-CTFγ to the production of amyloid β protein peptides is not known, we conclude that in contrast to the highly selective PS-dependent processing of Notch, the PS-dependent γ-secretase processing of APP is largely nonselective, and occurs at multiple sites within the APP transmembrane domain.

3.218 Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes

Falguieres, T. et al

*Mol. Biol. Cell, **12**, 2453-2468 (2001)*

In HeLa cells, Shiga toxin B-subunit is transported from the plasma membrane to the endoplasmic reticulum, via early endosomes and the Golgi apparatus, circumventing the late endocytic pathway. We describe here that in cells derived from human monocytes, i.e., macrophages and dendritic cells, the B-subunit was internalized in a receptor-dependent manner, but retrograde transport to the biosynthetic/secretory pathway did not occur and part of the internalized protein was degraded in lysosomes. These differences correlated with the observation that the B-subunit associated with Triton X-100 resistant membranes in HeLa cells, but not in monocyte-derived cells, suggesting that retrograde targeting to the biosynthetic/secretory pathway required association with specialized microdomains of biological membranes. In agreement with this hypothesis we found that in HeLa cells, the B-subunit resisted extraction by Triton X-100 until its arrival in the target compartments of the retrograde pathway, i.e., the Golgi apparatus and the endoplasmic reticulum. Furthermore, destabilization of Triton X-100-resistant membranes by cholesterol extraction potently inhibited B-subunit transport from early endosomes to the *trans*-Golgi network, whereas under the same conditions, recycling of transferrin was not affected. Our data thus provide first evidence for a role of lipid asymmetry in membrane sorting at the interface between early endosomes and the *trans*-Golgi network.

3.219 C-terminal domain of the Epstein-Barr virus LMP2A membrane protein contains a clustering signal

Matskova, L., Ernberg, I., Pawson, T. and Winberg, G.

*J. Virol., **75(22)**, 10941-10949 (2001)*

The latency-regulated transmembrane protein LMP2A interferes with signaling from the B-cell antigen receptor by recruiting the tyrosine kinases Lyn and Syk and by targeting them for degradation by binding the cellular E3 ubiquitin ligase AIP4. It has been hypothesized that this constitutive activity of LMP2A requires clustering in the membrane, but molecular evidence for this has been lacking. In the present study we show that LMP2A coclusters with chimeric rat CD2 transmembrane molecules carrying the 27-amino-acid (aa) intracellular C terminus of LMP2A and that this C-terminal domain fused to the glutathione-S-transferase protein associates with LMP2A in cell lysates. This molecular association requires neither the cysteine-rich region between aa 471 and 480 nor the terminal three aa 495 to 497. We also show that the juxtamembrane cysteine repeats in the LMP2A C terminus are the major targets for palmitoylation but that this acylation is not required for targeting of LMP2A to detergent-insoluble glycolipids-enriched membrane microdomains.

3.220 Agonist-promoted trafficking of human bradykinin receptors: arrestin- and dynamin-independent sequestration of the B_2 receptor and bradykinin in HEK293 cells

Lamb, M.E., de Weerd, W.F.C. and Leeb-Lundberg, L.M.F.

*Biochem. J., **355**, 741-750 (2001)*

In this study, we analyzed the agonist trafficking of human B_2 (B_2R) and B_1 (B_1R) bradykinin (BK) receptors using wild-type and green fluorescent protein (GFP)-tagged receptors in HEKL293 cells. B_2R was sequestered to a major extent upon exposure to BK, as determined by the loss of cell-surface B_2R using radioligand binding and by imaging of B_2R-GFP using laser-scanning confocal fluorescence microscopy. Concurrent BK sequestration was revealed by the appearance of acid-resistant specific BK receptor binding. The same techniques showed that B_1R was sequestered to a considerably lesser extent upon binding of des-Arg^{10}-kallidin. B_2R sequestration was rapid (half-life ~ 5 min) and reached a steady-state level that was significantly lower than that of BK sequestration. B_2R sequestration was minimally inhibited by K44A dynamin (22.4 ± 3.7%), and was insensitive to arrestin- (319-418), which are dominant-negative mutants of dynamin I and β-arrestin respectively. Furthermore, the B_2R-mediated sequestration of BK was completely insensitive to both mutants, as well as the association of BK with a caveolae-enriched fraction of the cells. On the other hand, agonist-promoted sequestration of the β_2-adrenergic receptor was dramatically inhibited by K44A dynamin (81.2 ± 16.3%) and by arrestin- (319-418) (36.9 ± 4.4%). Our results show that B_2R is sequestered to a significantly greater extent than is B_1R upon agonist treatment in HEK293 cells. Furthermore, B_2R appears to be recycled in the process of sequestering BK, and this process occurs in a dynamin- and β-arrestin-independent manner and, at least in part, involves caveolae.

3.221 Subcellular site of superoxide dismutase expression differentially controls AP-1 activity and injury in mouse liver following ischemia/reperfusion

Zhou, W. et al

Hepatology, ***33***, *902-914 (2001)*

Acute damage following ischemia and reperfusion (I/R) in the liver is in part caused by the generation of reactive oxygen species, such as superoxides, during the reperfusion event. Gene therapy directed at attenuating mitochondrial superoxide production following warm I/R injury in the liver has demonstrated great promise in reducing acute hepatocellular damage. In the present study, we have compared the therapeutic effects of ectopic expression of mitochondrial (MnSOD) and cytoplasmic (Cu/ZnSOD) superoxide dismutase using recombinant adenoviral vectors for reducing I/R damage in the liver. Consistent with previous observations, recombinant adenoviral delivery of MnSOD to the liver significantly attenuated both acute liver damage and AP-1 activation following I/R injury to the livers of mice. However, ectopic expression of Cu/ZnSOD diminished neither I/R-induced elevations in serum alanine transaminase (ALT) nor AP-1 activation. Interestingly, baseline activation of AP-1 before I/R-induced injury was seen in livers infected with recombinant Ad.Cu/ZnSOD, but not Ad.MnSOD or Ad.LacZ, vectors. The level of Cu/ZnSOD-induced AP-1 activation was significantly reduced by ablation of Kupffer cells or by coexpression of catalase, suggesting that increased H_2O_2 production facilitated by Cu/ZnSOD in hepatocytes and/or Kupffer cells may be responsible for AP-1 activation. *In vitro* reconstitution studies using hepatocyte and macrophage cell lines demonstrated that Cu/ZnSOD overexpression induces AP-1 in both cell types, and that secretion of a Cu/ZnSOD-induced macrophage factor is capable of elevating AP-1 in hepatocytes. In summary, our findings demonstrate that subcellular sites of superoxide production in the liver can differentially affect the outcome.

3.222 Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion

Ishihara, N. et al

Mol. Biol. Cell, ***12***, *3690-3702 (2001)*

Double membrane structure, autophagosomes, is formed *de novo* in the process of autophagy in the yeast *Saccharomyces cerevisiae*, and many Apg proteins participate in the process. To further understand autophagy, we analyzed the involvement of factors engaged in the secretory pathway. First, we showed that Sec18p (N-ethylmaleimide-sensitive fusion protein, NSF) and Vti1p (soluble N-ethylmaleimide-sensitive fusion protein attachment protein, SNARE), and soluble N-ethylmaleimide-sensitive fusion protein receptor are required for fusion of the autophagosomes to the vacuole but are not involved in autophagosome formation. Second, Sec12p was shown to be essential for autophagy but not for the cytoplasm to vacuole-targeting (Cvt) pathway, which shares mostly the same machinery with autophagy. Subcellular fractionation and electron microscopic analyses showed that Cvt vesicles, but not autophagosomes, can be formed in *sec12* cells. Three other coatmer protein (COPII) mutants, *sec16*, *sec23*, and *sec24*, were also defective in autophagy. The blockage of autophagy in these mutants was not dependent on transport from endoplasmic reticulum-to-Golgi, because mutations in two other COPII genes, *SEC13* and *SEC31*, did not affect autophagy. These results demonstrate the requirement for subgroup of COPII proteins in autophagy. This evidence demonstrating the involvement of Sec proteins in the mechanism of autophagosome formation is crucial for understanding membrane flow during the process.

3.223 ***Yarrowia lipolytica* Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex 21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway**
Einwachter, H., Sowinski, S., Kunau, W-H. and Schliebs, W.
*EMBO Reports, **2(11)**, 1035-1039 (2001)*

Import of peroxisomal matrix proteins is essential for peroxisome biogenesis. Genetic and biochemical studies using a variety of different model systems have led to the discovery of 23 *PEX* genes required for this process. Although it is generally believed that, in contrast to mitochondria and chloroplasts, translocation of proteins into peroxisomes involves a receptor cycle, there are reported differences of an evolutionary conservation of this cycle either with respect to the components or the steps involved in different organisms. We show here that the early steps of protein import into peroxisomes exhibit a greater similarity than was thought previously to be the case. Pex20p of *Yarrowia lipolytica*, Pex18p and Pex21p of *Saccharomyces cerevisiae* and mammalian Pex5pL fulfill a common function in the PTS2 pathway of their respective organisms. These non-orthologous proteins possess a conserved sequence region that most likely represents a common PTS2-receptor binding site and di-aromatic pentapeptide motifs that could be involved in binding of the putative docking proteins. We propose that no necessarily the same proteins but functional modules of them are conserved in the early steps of peroxisomal protein import.

3.224 **Sec6/8 complexes on trans-Golgi network and plasma membrane regulate stages of exocytosis in mammalian cells**
Yeaman, C., Grindstaff, K.K., Wright, J.R. and Nelson, W.J.
*J. Cell Biol., **155(12)**, 593-604 (2001)*

Sec6/8 complex regulates delivery of exocytic vesicles to plasma membrane docking sites, but how it is recruited to specific sites in the exocytic pathway is poorly understood. We identified a Sec6/8 complex on trans-Golgi network (TGN) and plasma membrane in normal rat kidney (NRK) cells that formed either fibroblast- (NRK-49F) or epithelial-like (NRK-52E) intercellular junctions. At both TGN and plasma membrane, Sec6/8 complex colocalizes with exocytic cargo protein, vesicular stomatitis virus G protein (VSVG)-tsO45. Newly synthesized Sec6/8 complex is simultaneously recruited from the cytosol to both sites. However, brefeldin A treatment inhibits recruitment to the plasma membrane and other treatments that block exocytosis (e.g., expression of kinase-inactive protein kinase D and low temperature incubation) cause accumulation of Sec6/8 on the TGN, indicating that steady-state distribution of Sec6/8 complex depends on continuous exocytic vesicle trafficking. Addition of antibodies specific for TGN- or plasma membrane-bound Sec6/8 complexes to semiintact NRK cells results in cargo accumulation in a perinuclear region or near the plasma membrane, respectively. These results indicate that Sec6/8 complex is required for several steps in exocytic transport of vesicles between TGN and plasma membrane.

3.225 **Open reading frame III of Borna disease virus encodes a nonglycosylated matrix protein**
Kraus, I et al
*J. Virol., **75(24)**, 12098-12104 (2001)*

The open reading frame III of Borna virus (BDV) codes for a protein with a mass of 16kDa, named p16 or BDV-M. p16 was described as an N-glycosylated protein in several previous publications and therefore was termed gp18, although the amino acid sequence of p16 does not contain any regular consensus sequence for N glycosylation. We examined glycosylation of p16 and studied its membrane topology using antisera raised against peptides, which comprise the N and the C termini. Neither an N- nor a C-terminal peptide is cleaved from p16 during maturation. Neither deglycosylation of p16 by endoglycosidases nor binding of lectin to p16 was detectable. Introduction of typical N-glycosylation sites at the proposed sites of p16 failed in carbohydrate attachment. Flotation experiments with membranes of BDV-infected cells on density gradients revealed that p16 is not an integral membrane protein, since it can be dissociated from membranes. Our experimental data strongly suggest that p16 is a typical nonglycosylated matrix protein associated at the inner surface of the viral membrane, as is true for homologous protein of other members of the *Mononegavirales* order.

3.226 Association of Na^+ -H^+ exchanger isoform NHE3 and dipeptidyl peptidase IV in the renal proximal tubule

Girardi, A.C.C., Degray, B.C., Nagy, T., Biemesderfer, D. and Aronsen, P.
J. Biol. Chem., ***276(49)***, *46671-46677 (2001)*

In an attempt to identify proteins that assemble with the apical membrane Na^+ -H^+ exchanger isoform NHE3, we generated monoclonal antibodies (mAbs) against affinity-purified NHE3 protein complexes isolated from solubilized renal microvillus membrane vesicles. Hybridomas were selected based on their ability to immunoprecipitate NHE3. We have characterized in detail one of the mAbs (1D11) that specifically co-precipitated NHE3 but not villin or NaPi-2. Western blot analyses of microvillus membranes and immunoelectron microscopy of kidney sections showed that mAb 1D11 recognizes a 110-kDa protein highly expressed on the apical membrane of proximal tubule cells. Immunoaffinity chromatography was used to isolate the antigen against which mAb 1D11 is directed. N-terminal sequencing of the purified protein identified it as dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.15), which was confirmed by assays of DPPIV enzyme activity. We also evaluated the distribution of the NHE3-DPPIV complex in microdomains of rabbit renal brush border. In contrast to the previously described NHE3-megalin complex, which principally resides in a dense membrane population (coated pits) in which NHE3 is active, the NHE3-DPPIV complex was predominantly in the microvillar fraction in which NHE3 is active. Serial precipitation experiments confirmed that anti-megalin and anti-DPPIV antibodies co-precipitate different pools of NHE3. Taken together, these studies revealed an unexpected association of the brush border Na^+ -H^+ exchanger NHE3 with dipeptidyl peptidase IV in the proximal tubule. These findings raise the possibility that association with DP-PIV may affect NHE3 surface expression and/or activity.

3.227 Evidence for coupling of membrane targeting and function of the signal recognition particle (SRP) receptor FtsY

Herskovits, A.A. et al
*EMBO Reports, **2(11),** 1040-1046 (2001)*

Recent studies have indicated that FtsY, the signal recognition particle receptor of *Escherichia coli*, plays a central role in membrane protein biogenesis. For proper function, FtsY must be targeted to the membrane, but its membrane-targeting pathway is unknown. We investigated the relationship between targeting and function of FtsY *in vivo*, by separating its catalytic domain (NG) from its putative targeting domain (A) by three means: expression of split *ftsY*, insertion of various spacers between A and NG, and separation of A and NG by the *in vivo* proteolysis. Proteolytic separation of A and NG does not abolish function, whereas separation by long linkers or expression of split *ftsY* is detrimental. We propose that proteolytic cleavage of FtsY occurs after completion of co-translational targeting and membrane assembly of NG. In contrast, separation by other means may interrupt proper synchronization of co-translational targeting and membrane assembly of NG. The co-translational interaction of FtsY with the membrane was confirmed by *in vitro* experiments.

3.228 Direct evidence for a two-step assembly of apoB48-containing lipoproteins in the lumen of the smooth endoplasmic reticulum of rabbit enterocytes

Cartwright, I.J. and Higgins, J.A.
J. Biol. Chem., ***276(51)***, *48048-48057 (2001)*

The aim of this study was to investigate the types and characteristics of chylomicrons precursor in the lumen of the secretory compartment of rabbit enterocytes. Luminal contents were separated into density subfractions in two continuous self-generating gradients of different density profiles. In enterocytes from rabbits fed a low fat diet, newly synthesized and immunodetectable apoB48 was only in the subfraction of density similar to high density lipoprotein (dense particles); the luminal triacylglycerol (TAG) content was low and only in the subfraction of density similar to that of chylomicrons/very low density lipoproteins (light particles). After feeding fat, newly synthesized and immunodetectable apoB48 was in both dense (phospholipid-rich) and light (TAG-rich) particles. Luminal TAG mass and synthesis increased after fat feeding and was only in light particles. Pulse-chase experiments showed that the luminal-radiolabeled apoB48 lost from the dense particles was recovered in the light particles and the secreted chylomicrons. All of the light particle lipids (mass and newly synthesized) co-immunoprecipitated with apoB48. However, in the dense particles, there was a preferential co-precipitation of the preexisting rather than newly synthesized phospholipid. Assembly of apoB48-containing TAG-enriched lipoproteins is therefore a two-step process. The first step produces dense apoB48 phospholipid-rich particles, which accumulate in

the smooth endoplasmic reticulum lumen. In the second step, these dense particles rapidly acquire the bulk of the TAG and additional phospholipid in a single and rapid step.

3.229 Quantitative and reproducible two-dimensional gel analysis using Phoretix 2D Full
Mahon, P. and Dupree, P.
*Electrophoresis, **22**, 2075-2085 (2001)*

Quantitative two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is used to determine changes in individual protein levels in complex protein mixtures. To provide reliable data, the software used for 2-D gel image analysis must provide a linear response over a wide dynamic range of data output. Here, we show that Phoretix 2D Full analysis of 2-D gels stained with colloidal Coomassie Brilliant Blue G-250 can provide a linear measure of changes in protein quantity. We show using a complex mixture of *Arabidopsis thaliana proteins*, that this is true for essentially all focused proteins, in a data output range greater than three orders of magnitude. An analysis of the factors that affect errors in the results demonstrated that reproducibility of the data is significantly improved by user seeding, whereas it is reduced by use of the background subfraction algorithms.

3.230 Cvt18/Gsa12 is required for cytoplasm-to-vacuole transport, pexophagy, and autophagy in *Saccharomyces cerevisiae* and *Pichia pastoris*
Guan, J. et al
*Mol. Biol. Cell, **12(12)**, 3821-3838 (2001)*

Eukaryotic cells have the ability to degrade proteins and organelles by selective and nonselective modes of micro- and macroautophagy. In addition, there exist both constitutive and regulated forms of autophagy. For example, pexophagy is a selective process for the regulated degradation of peroxisomes by autophagy. Our studies have shown that the differing pathways of autophagy have many molecular events in common. In this article, we have identified a new member in the family of autophagy genes. *GSA12* in *Pichia pastoris* and its *Saccharomyces cerevisiae* counterpart, *CVT18*, encode a soluble protein with two WD40 domains. We have shown that these proteins are required for pexophagy and autophagy in *P. pastoris* and the Cvt pathway, autophagy, and pexophagy in *S. cerevisiae*. In *P. pastoris*, Gsa12 appears to be required for an early event in pexophagy. That is, the involution of the vacuole or extension of vacuole arms to engulf the peroxisomes does not occur in the *gsa12* mutant. Consistent with its role in vacuole engulfment, we have found that this cytosolic protein is also localized to the vacuole surface. Similarly, Cvt18 displays a subcellular localization that distinguishes it from the characterized proteins required for cytoplasm-to-vacuole delivery pathways.

3.231 Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast
Bagnat, M., Chang, A. and Simons, K.
*Mol. Biol. Cell, **12(12)**, 4129-4138 (2001)*

Correct sorting of proteins is essential to generate and maintain the identity and function of the different cellular compartments. In this study we demonstrate the role of lipid rafts in biosynthetic delivery of Pma1p, the major plasma membrane proton ATPase, to the cell surface. Disruption of rafts led to mistargeting of Pma1p to the vacuole. Conversely, Pma1-7, an ATPase mutant that is mistargeted to the vacuole, was shown to exhibit impaired raft association. One of the previously identified suppressors, multicopy *AST1*, not only restored surface delivery but also raft association of Pma1-7. Ast1p, which is a peripheral membrane protein, was found to directly interact with Pma1p inducing its clustering into a SDS/Triton X100-resistant oligomer. We suggest that clustering facilitates partition of Pma1p into rafts and transport to cell surface.

3.232 Na^+-H^+ exchanger 3 (NHE3) is present in lipid rafts in the rabbit ileal brush border: a role for rafts in trafficking and rapid stimulation of NHE3
Li, X et al
*J. Physiol., **537(2)**, 537-552 (2001)*

Rabbit ileal Na^+-absorbing cell Na^+-H^+ exchanger 3 (NHE3) was shown to exist in three pools in the brush border (BB), including a population in lipid rafts. Approximately 50% of BB NHE3 was associated with Triton X-100-soluble fractions and the other ~50% with Triton X-100-insoluble fractions; ~33% of the detergent-insoluble NHE3 was present in cholesterol-enriched lipid microdomains (rafts).

The raft pool of NHE3 was involved in the stimulation of BB NHE3 activity with epidermal growth factor (EGF). Both EGF and clonidine treatments were associated with a rapid increase in the total amount of BB NHE3. This EGF- and clonidine-induced increase of BB NHE3 was associated with an increase in the raft pool of NHE3 and to a smaller extent with an increase in the total detergent-insoluble fraction, but there was no change in the detergent-soluble pool. In agreement with the rapid increase in the amount of NHE3 in the BB, EGF also caused a rapid stimulation of BB Na^+-H^+ exchange activity.

Disrupting rafts by removal of cholesterol with methyl-β-cyclodextrin (MβCD) or destabilizing the actin cytoskeleton with cytochalasin D decreased the amount of NHE3 in early endosomes isolated by OptiPrep gradient fractionation. Specifically, NHE3 was shown to associate with endosomal vesicles immunoisolated by anti-EEA1 (early endosomal autoantigen 1) antibody-coated magnetic beads and the endosome-associated NHE3 was decreased by cytochalasin D and MβCD treatment.

We conclude that (i) a pool of ileal BB NHE3 exists in lipid rafts; (ii) EGF and clonidine increase the amount of BB NHE3; (iii)lipid rafts and to a lesser extent, the cytoskeleton, but not the detergent-soluble NHE3 pool, are involved in the EGF- and clonidine-induced acute increase in amount of BB NHE3; (iv) lipid rafts and the actin cytoskeleton play important roles in the basal endocytosis of BB NHE3.

3.233 *YFH1*-mediated iron homeostasis is independent of mitochondrial respiration
Chen, O.S. and Kaplan, J.
*FEBS Lett., **509**, 131-134 (2001)*

The human gene frataxin and its yeast homolog *YFH1* affect mitochondrial function. Deficits in frataxin result in Friedreich ataxia, while deletion of *YFH1* results in respiratory incompetence. We determined that as long as respiratory incompetent yeast express Yfh1p they do not accumulate excessive mitochondrial iron. Deletion of *YFH1* in respiratory incompetent yeast results in mitochondrial iron accumulation, while the reintroduction of Yfh1p results in mitochondrial iron export. Further, overexpression of Yfh1p has no effect on oxygen consumption in wild-type yeast grown in either fermentative or respiratory carbon sources. We conclude that the effect of Yfh1p on mitochondrial iron metabolism is independent of respiratory activity.

3.234 Subcellular localization of presenilin 2 endoproteolytic C-terminal fragments
Tekirian, T.L. et al
*Mol. Brain Res., **96**, 14-20 (2001)*

Mutations in the genes that encode the presenilin 1 and 2 (PS1 and PS2) proteins cause the majority of familial Alzheimer's disease (FAD). Differential cleavage of the presenilins results in a generation of at least two C-terminal fragments (CTFs). An increase in the smaller of these two CTFs is one of the few changes in presenilin processing associated with FAD mutations in both PS1 and PS2. Interestingly, the phosphorylation of PS2 modulates the production of the smaller, caspase-derived PS2 CTF, which indicates that the generation of this fragment is a regulated physiologic event. To date, there is no data concerning the subcellular distribution of the caspase-derived PS2 CTF. Because this fragment is normally present at levels that are difficult to detect, we have used cell lines in which the production of wild-type or N141I mutant PS2 is controlled by a tetracycline-regulated promoter in order to assess the subcellular localization of the caspase CTF in relation to the larger, constitutive PS2 CTF and to PS2 holoprotein. We have found that when levels of PS2 are low, the constitutive CTF colocalizes with markers consistent with localization in the early Golgi-ER-Golgi intermediate compartment (ERGIC) while the caspase CTF colocalizes with markers for the endoplasmic reticulum (ER). Following induction of wild-type or mutant PS2, when the levels of PS2 are high, the primary localization of the constitutive CTF appears to shift from the early Golgi-ERGIC in addition to the ER. Interestingly, while the induction of wild-type PS2 resulted in the localization of the caspase CTF primarily in the ER, the induction of mutant PS2 resulted in the localization of the caspase CTF to both the ER and the early Golgi-ERGIC. In summary, these data suggest that the two presenilin 2 CTFs have different patterns of subcellular localization and that the N141I PS2 mutation alters the localization pattern of the PS2 caspase fragment.

3.235 The role of caveolae and caveolin in vesicle-dependent and vesicle-independent trafficking
Matveev, S., Li, X., Everson, W. and Smart, E.J.
*Adv. Drug. Deliver. Rew., **49**, 237-240 (2001)*

Caveolae can mediate endocytosis, transcytosis, and potocytosis. Our understanding of these processes as well as the elucidation of the molecular machinery involved has greatly expanded. In addition, caveolin, a 22 kDa protein often associated with caveolae, can promote the trafficking of sterol through the cytoplasm independent of vesicles. Caveolin also influences the formation, morphology, and function of caveolae. The ability of caveolae and caveolin to mediate macromolecular transport directly impacts a variety of physiological and pathophysiological processes.

3.236 Localization of p24 putative cargo receptors in the early secretory pathway depends on the biosynthetic activity of the cell
Kuiper, R.P. et al
*Biochem. J., **360**, 421-429 (2001)*

Members of the p24 family of putative cargo receptors (subdivided into p24-α, -β, -γ and -δ) are localized in the intermediate- and cis-Golgi compartment of the early secretory pathway, and are thought to play an important role in protein transport. In the present study, we wondered what effect increased biosynthetic cell activity with resulting high levels of protein transport would have on the subcellular localization of p24. We examined p24 localization in *Xenopus* intermediate pituitary melanotrope cells, which in black- and white-adapted animals are biosynthetically highly active and virtually inactive respectively. In addition, p24 localization was studied in *Xenopus* anterior pituitary cells whose activity is not changed during background adaptation. Using organelle fractionation, we found that in the inactive melanotropes and moderately active anterior pituitary cells of white-adapted animals, the p24-α, -β, -γ and -δ proteins are all located in the Golgi compartment. In the highly active melanotropes, but not in the anterior cells of black-adapted animals, the steady-state distribution of all four p24 members changed towards the intermediated compartment and subdomains of the endoplasmic reticulum (ER), most probably the ER exit sites. In the active melanotropes, the major cargo protein pro-opiomelanocortin was mostly localized to ER subdomains and partially co-localized with the p24 proteins. Furthermore, in the active cells, *in vitro* blocking of protein biosynthesis by cycloheximide or dispersion of the Golgi complex by brefeldin A led to a redistribution of the p24 proteins, indicating their involvement in ER-to-Golgi protein transport and extensive cycling in the early secretory pathway. We conclude that the subcellular localization of p24 proteins is dynamic and depends on the biosynthetic activity of the cell.

3.237 Preptin derived from proinsulin-like growth factor II (proIGF-II) is secreted from pancreatic islet β-cells and enhances insulin secretion
Buchanan, C.M., Phillips, A.R. and Cooper, G.J.S.
*Biochem. J., **360**, 431-439 (2001)*

Pancreatic islet β-cells secrete the hormones insulin, amylin and pancreastatin. To search for further β-cell hormones, we purified peptides from secretory granules isolated from cultured murine βTC6-F7 β-cells. We identified a 34 amino-acid peptide (3948 Da), corresponding to Asp^{69}-Leu^{102} of the proinsulin-like growth factor II E-peptide, which we have termed "preptin". Preptin is present in islet β-cells and undergoes glucose-mediated co-secretion with insulin. Synthetic preptin increase insulin secretion from glucose-stimulated βTC6-F7 cells in a concentration-dependent and saturable manner. Preptin infusion into the isolated, perfused rat pancreas increases the second phase of glucose-mediated insulin secretion by 30%, while antipreptin immunoglobulin infusion decreases the first and second phases of insulin secretion by 29 and 26% respectively. These findings suggest that preptin is a physiological amplifier of glucose-mediated insulin secretion.

3.238 Ubiquitin sorts proteins into the intralumenal degradative compartment of the late-endosome/vacuole
Urbanowski, J.L. and Piper, R.C.
*Traffic, **2**, 622-630 (2001)*

Many studies have demonstrated a role for ubiquitin (Ub) in the down-regulation of cell surface proteins. In yeast, down-regulation is marked by the internalization of proteins, followed by their delivery to the lumen of the vacuole where both the cytosolic and lumenal domains are degraded. It is generally believed that the regulatory step of this process is internalization from the plasma membrane and that protein delivery to the lysosome or vacuole is by default. By separating the process of internalization from degradation, we demonstrate that incorporation of proteins into intralumenal vesicles represents a distinct sorting step along the endocytic pathway that is controlled by recognition of ubiquitin. We show that attachment of a single ubiquitin can serve as a specific sorting signal for the degradative pathway by

redirecting recycling Golgi proteins and resident vacuolar proteins into intralumenal vesicles of the yeast vacuole. This pathway is independent of Ptdlns (3,5) P2 and does not rely on the specific composition of transmembrane domain segments. These data provide a physiological basis for how ubiquitination of cell surface proteins guides their degradation and removal from the recycling pathway.

3.239 Membrane protein diffusion sets the speed of rod phototransduction
Calvert, P.D. et al
*Nature, **411**, 90-94 (2001)*

Retinal rods signal the activation of a single receptor molecule by a photon. To ensure efficient photon capture, rods maintain about 10^9 copies of rhodopsin densely packed into membranous disks. But a high packing density of rhodopsin may impede other steps in phototransduction that take place on the disk membrane, by restricting the lateral movement of, and hence the rate of encounters between, the molecules involved. Although it has been suggested that lateral diffusion of proteins on the membrane sets the rate of onset of the photoresponse, it was later argued that the subsequent processing of the complexes was the main determinant of this rate. The effects of protein density on response shut-off have not been reported. Here we show that a roughly 50% reduction in protein crowding achieved by the hemizygous knockout of rhodopsin in transgenic mice accelerates the rising phases and recoveries of flash responses by about 1.7-fold *in vivo*. Thus, in rods the rates of both response onset and recovery are set by the diffusional encounter frequency between proteins on the disk membrane.

3.240 Kinesin-mediated axonal transport of a membrane compartment containing β-secretase and presenilin-1 requires APP
Kamal, A. et al
*Nature, **414**, 643-648 (2001)*

Proteolytic processing of amyloid precursor protein (APP) generates amyloid-β peptide and has been implicated in the pathogenesis of Alzheimer's disease. However, the normal function of APP, whether this function is related to the proteolytic processing of APP, and where this processing takes place in neurons *in vivo* remain unknown. We have previously shown that the axonal transport of APP in neurons is mediated by the direct binding of APP to the kinesin light chain subunit of kinesin-I, a microtubule motor protein. Here we identify an axonal membrane compartment that contains APP, β-secretase and presenilin-1. The fast anterograde axonal transport of this compartment is mediated by APP and kinesin-I. Proteolytic processing of APP can occur in the compartment *in vitro* and *in vivo* in axons. This proteolysis generates amyloid-β and a carboxy-terminal fragment of APP, and liberates kinesin-I from the membrane. These results suggest that APP functions as a kinesin-I membrane receptor, mediating the axonal transport of β-secretase and presenilin-1, and that processing of APP to amyloid-β by secretases can occur in an axonal membrane compartment transported by kinesin-I.

3.241 Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid β-peptide
Puglielli, L. et al
*Nature Cell Biol., **3**, 905-912 (2001)*

The pathogenic event common to all forms of Alzheimer’s disease is the abnormal accumulation of the amyloid β-peptide (Aβ). Here we provide strong evidence that intracellular cholesterol compartmentation modulates the generation of Aβ. Using genetic, biochemical and metabolic approaches, we found that cholesteryl-ester levels are directly correlated with Aβ production. Acyl-coenzyme A: cholesterol acyltransferase (ACAT), the enzyme that catalyses the formation of cholesteryl esters, modulates the generation of Aβ through the tight control of the equilibrium between free cholesterol and cholesteryl esters. We also show that pharmacological inhibitors of ACAT, developed for the treatment of atherosclerosis, are potent modulators of Aβ generation, indicating their potential for use in the treatment of Alzheimer’s disease.

3.242 Caspase-3 is localized to endothelial caveolar domains
Oxhorn, B.C., Wadia, R. and Buxton, I.L.O.
*Proc. West. Pharmacol. Soc., **44**, 45-48 (2001)*

No abstract available

3.243 Vesicle Permeabilization by Protofibrillar α-Synuclein: Implications for the Pathogenesis and Treatment of Parkinson's Disease
Volles, M.J. et al
Biochemistry, ***40(26)***, *7812-7819 (2001)*

Fibrillar α-synuclein is a component of the Lewy body, the characteristic neuronal inclusion of the Parkinson's disease (PD) brain. Both α-synuclein mutations linked to autosomal dominant early-onset forms of PD promote the in vitro conversion of the natively unfolded protein into ordered prefibrillar oligomers, suggesting that these protofibrils, rather than the fibril itself, may induce cell death. We report here that protofibrils differ markedly from fibrils with respect to their interactions with synthetic membranes. Protofibrillar α-synuclein, in contrast to the monomeric and the fibrillar forms, binds synthetic vesicles very tightly via a β-sheet-rich structure and transiently permeabilizes these vesicles. The destruction of vesicular membranes by protofibrillar α-synuclein was directly observed by atomic force microscopy. The possibility that the toxicity of α-synuclein fibrillization may derive from an oligomeric intermediate, rather than the fibril, has implications regarding the design of therapeutics for PD.

3.244 The clustered FcγR receptor II is recruited to Lyn-containing membrane domains and undergoes phosphorylation in a cholesterol-dependent manner
Kwiatkowska, K. and Sobota, A.
Eur. J. Immunol., ***31(4)***, *989-998 (2001)*

Phosphorylation of clustered FcγR receptor II (FcγRII) by Src family tyrosine kinases is the earliest event in the receptor signaling cascade. However, the molecular mechanisms for the interaction between FcγRII and these kinases are not elucidated. To asses this problem we isolated high molecular weight complexes of cross-linked FcγRII from non-ionic detergent lysates of U937 monocytic cells. CD55, a glycosylphosphatidylinositol-anchored protein, a ganglioside GM1 and Lyn, a Src family tyrosine kinase, were also located in these complexes. Gradient centrifugation demonstrated that the complexes containing cross-linked FcγRII displayed a low buoyant density. The FcγRII present in the complexes underwent tyrosine phosphorylation. Cross-linked FcγRII and Lyn occupied common 100–200 nm detergent-resistant membrane fragments, as demonstrated by immunoprecipitation and microscopy studies. Pretreatment of the cells with β-cyclodextrin, a cholesterol acceptor, depleted membrane cholesterol and released CD55, GM1 and Lyn from the detergent-resistant complexes. In parallel, the association of Lyn with cross-linked FcγRII was disrupted and phosphorylation of the receptor inhibited. Reincorporation of cholesterol evoked the relocation of Lyn into the detergent-resistant membrane fraction and restored both Lyn association with cross-linked FcγRII and tyrosine phosphorylation of the receptor. Our data demonstrate that cholesterol-enriched membrane rafts can facilitate tyrosine phosphorylation of clustered FcγRII by Lyn kinase.

3.245 The influence of CD40 on the association of the B cell antigen receptor with lipid rafts in mature and immature cells
Malapati, S. and Pierce, S.K.
Eur. J. Immunol., ***31(12)***, *3789-3797 (2001)*

Cholesterol- and sphingolipid-rich membrane microdomains termed lipid rafts appear to play a central role in B cell activation. In mature B cells, signaling through the B cell antigen receptor(BCR) is initiated from within rafts and leads to activation. In immature B cells, the BCR is excluded from rafts and signaling leads to apoptosis. CD40, a member of the tumor necrosis receptor family, is expressed by B cells throughout development and has been shown to influence the results of the engagement of antigen by the BCR in both mature B and immature B cells. Here evidence is provided that CD40 is excluded from the lipid rafts of both mature and immature B cells and remains excluded from rafts even after cross-linking. Nevertheless, in mature B cells CD40 signaling influences the association of the BCR with rafts resulting in an increase in the amount of BCR that translocates into rafts following ligand binding and a subsequent acceleration of the movement of the BCR from rafts. In immature B cells, the cross-linked BCR remains excluded from rafts in the presence of CD40 signaling, conditions under which BCR-induced apoptosis is blocked. These results indicate that CD40 functions outside lipid rafts to influence raft-dependent events in mature B cells and raft-independent events in immature B cells.

3.246 Mechanisms of Aβ Production and Aβ Degradation: Routes to the Treatment of Alzheimer's Disease
Selkoe, D.J., Xia, W., Kimberly, W.T., Vekrellis, K., Walsh, D., Esler, W.P. and Wolfe, M.S:
Alzheimer's Disease: Advances in Ethiology, Pathogenesis and Therapeutics, 421-432 (2001)

In this chapter, we summarize recent work from our laboratories on three interrelated aspects of the role of amyloid β-protein as the initiator of Alzheimer's disease: its production, degradation and aggregation. By analyzing each of these three steps in the economy of Beta-amyloid independently and then attempting to bring together the findings into a unified cycle, we hope to shed light, not only on multiple mechanisms by which Beta-amyloid can accumulate to induce neuronal dysfunction, but also on discrete points of therapeutic intervention.

3.247 Convergence of multiple autophagy and cytoplasm to vacuole components to a perivacuolar membrane compartment prior to *de novo* vesicle formation
Kim, J., Huang, W-P., Stromhaug, P.E. and Klionsky, D.J.
J. Biol. Chem., ***277(1)****, 763-773 (2002)*

Under starvation conditions, the majority of intracellular degradation occurs at the lysosome or vacuole by the autophagy pathway. The cytoplasmic substrates destined for degradation are packaged inside unique double-membrane transport vesicles called autophagosomes and targeted to the lysosome/vacuole for subsequent breakdown and recycling. Genetic analyses of yeast autophagy mutants, *apg* and *aut*, have begun to identify to molecular machinery as well as indicate a substantial overlap with the biosynthetic cytoplasm to vacuole targeting (Cvt) pathway. Transport vesicle formation is a key regulatory step of both pathways. In this study, we characterize the putative compartment from which both autophagosomes and the analogous Cvt vesicles may originate. Microscopy analyses identified a perivacuolar membrane as the resident compartment for both the Apg1-Cvt9 signaling complex, which mediates the switching between autophagic and Cvt transport, and the autophagy/Cvt-specific phosphatidylinositol 3-kinase complex. Further, the perivacuolar compartment designates the initial site of membrane binding by the Apg/Cvt vesicle component Aut7, the Cvt cargo receptor Cvt19, and the Apg conjugation machinery, which functions in the *de novo* formation of vesicles. Biochemical isolation of the vesicle component Aut7 and density gradient analyses recapitulate the microscopy findings while also supporting the paradigm that components required for vesicle formation and packaging concentrate at subdomains within the donor membrane compartment.

3.248 Membrane-bound α-synuclein has a high aggregation propensity and the ability to seed the aggregation of cytosolic form
Lee, H-J., Choi, C. and Lee, S-J.
J. Biol. Chem., ***277(1),*** *671-678 (2002)*

α-Synuclein exists as at least two structural isoforms: a helix-rich, membrane-bound form and a disordered, cytosolic form. Here, we investigated the role of membrane-bound α-synuclein in the aggregation process. In a cell-free system consisting of isolated brain fractions, spontaneous and progressive aggregation of α-synuclein was observed in membranes starting at day 1, whereas no aggregation was observed in the cytosolic fraction in a 3-day period. Addition of antioxidants reduced the aggregation in membrane fraction, implicating the role of oxidative modifications. When excess cytosolic α-synuclein was added to brain membranes, the rate of aggregation was increased while the lag-time was unaffected. Incorporation of cytosolic α-synuclein into membrane-associated aggregates was demonstrated by fractionation and coimmunoprecipitation experiments. In a previous study, we showed that mitochondrial inhibitors such as rotenone, induced α-synuclein aggregation in cells. In the present study using rotenone-treated cells, the earliest appearance of α-synuclein oligomeric species was observed in membranous compartments. Furthermore, α-synuclein-positive inclusions were co-stained with DiI, a membrane-partitioning fluorescent dye, confirming the presence of lipid components in α-synuclein aggregates. These results suggest that membrane-bound α-synuclein can generate nuclei that seed the aggregation of the more abundant cytosolic form.

3.249 Characterization of an acyl-CoA thioesterase that functions as a major regulator of peroxisomal lipid metabolism

Hunt, M.C., Solaas, K., Kase, B.F and Alexon, E.H.
*J. Biol. Chem., **277(2)**, 1128-1138 (2002)*

Peroxisomes function in β-oxidation of very long- and long-chain fatty acids, dicarboxylic fatty acids, bile acid intermediates, prostaglandins, leukotrienes, thromboxanes, pristanic acid and xenobiotic carboxylic acids. These lipids are mainly chain-shortened for excretion as the carboxylic acids are transported to mitochondria for further metabolism. Several of these carboxylic acids are slowly oxidized and may therefore sequester coenzyme A (CoASH). To prevent CoASH sequestration and to facilitate excretion of chain-shortened carboxylic acids, acyl-CoA thioesterases, which catalyze the hydrolysis of acyl-CoA to the free acid and CoASH, may play important roles. We have here cloned and characterized a peroxisomal acyl-CoA thioesterase from mouse, named PTE-2 (peroxisomal acyl-CoA thioesterase 2. PTE-2 is ubiquitously expressed and induced at mRNA level by treatment with the peroxisome proliferator WY-14,643 and fasting. Induction seen by these treatments was dependent on the peroxisome proliferator-activated receptor α. Recombinant PTE-2 showed a broad chain-length specificity with acyl-CoAs from short- and medium-, to long-chain acyl-CoAs, and other substrates including trihydroxycoprostanoyl-CoA, hydroxymethylglutaryl-CoA and branched chain acyl-CoAs, all of which are present in peroxisomes. Highest activities were found with the CoA esters of primary bile acids choloyl-CoA and chenodeoxycholoyl-CoA as substrates. PTE-2 activity is inhibited by free CoASH, suggesting that intraperoxisomal free CoASH levels regulate the activity of this enzyme. The acyl-CoA specificity of recombinant PTE-2 closely resembles that of purified mouse liver peroxisomes, suggesting that PTE-2 is the major acyl-CoA thioesterase in peroxisomes. Addition of recombinant PTE-2 to incubations containing isolated mouse liver peroxisomes strongly inhibited bile acid-CoA: amino acid N-acyltransferase activity, suggesting that this thioesterase can interfere with CoASH-dependent pathways. We propose that PTE-2 functions as a key regulator of peroxisomal lipid metabolism.

3.250 Dynamic association of human insulin receptor with lipid rafts in cells lacking caveolae

Vainio, S. et al
*EMBO Reports, **3(1)**, 1-6 (2002)*

Cholesterol-sphingolipid rich plasma membrane domains, known as rafts, have emerged as important regulators of signal transduction. The adipocytes insulin receptor (IR) is localized to and signals via caveolae that are formed by polymerization of caveolins. Caveolin binds to IR and stimulates signaling. We report that, in liver-derived cells lacking caveolae, autophosphorylation of the endogenous IR is dependent on raft lipids, being compromised by acute cyclodextrin-mediated cholesterol depletion or by antibody clustering of glycosphingolipids. Moreover, we provide evidence that IR becomes recruited to detergent-resistant domains upon ligand binding and that clustering of GM2 ganglioside inhibits IR signaling apparently by excluding the ligand-bound IR from these domains. Our results indicate that, in cells derived from liver, an important insulin target tissue, caveolae are not required for insulin signaling. Rather, the dynamic recruitment of the ligand-bound IR into rafts may serve to regulate interactions in the initiation of the IR signaling cascade.

3.251 Cholesteryl ester is transported from caveolae to internal membranes as part of a caveolin-annexin II lipid-protein

Uittenbogaard, A., Everson, W.V., Matveev, S.V. and Smart, E.J.
J. Biol. Chem., ***277(7)***, *4925-4931 (2002)*

We previously demonstrated that in Chinese hamster ovary cells scavenger receptor, class B, type I-dependent selective cholesteryl ester uptake occurs in caveolae. In the present study we hypothesized that cholesteryl ester is transported from caveolae through the cytosol to an internal membrane by a caveolin chaperon complex similar to the one we originally described for the transport of newly synthesized cholesterol. To test this hypothesis we incubated Chinese hamster ovary cells expressing scavenger receptor, class B, type I with (^{3}H)cholesteryl ester-labeled high density lipoprotein, subfractionated the cells and looked for a cytosolic pool of (^{3}H)cholesteryl ester. The radiolabeled sterol initially appeared in the caveolae fraction, then in the cytosol, and finally in the internal membrane fraction. Caveolin IgG precipitated all of the (^{3}H)cholesteryl ester associated with the cytosol. Co-immunoprecipitation studies demonstrated that in the presence of high density lipoprotein, but not low density lipoprotein or lipoprotein-deficient serum, caveolin IgG precipitated four proteins; annexin II, cyclophilin 40, caveolin, and cyclophilin A. Caveolin acylation-deficient mutants were used to demonstrate that acylation of cysteine 133 but not cysteine 143 or 156 is required for annexin II association with caveolin and the rapid transport of cholesteryl esters out of caveolae. We conclude that a caveolin-annexin II lipid-protein complex facilitates the rapid internalization of cholesteryl esters from caveolae.

3.252 Alzheimer's disease-related overexpression of the cation-dependent mannose-6-phosphate receptor increases Aβ secretion: Role for altered lysosomal hydrolase distribution in β-amyloidogenesis.

Mathews, PM et al
J. Biol. Chem., ***277(7)***, *5299-5307 (2002)*

Prominent endosomal and lysosomal changes are an invariant feature of neurons in sporadic Alzheimer's disease (AD). These changes include increased levels of lysosomal hydrolases in early endosomes and increased expression of the cation-dependent mannose 6-phosphate receptor (CD-MPR), which is partially localized to early endosomes. To determine whether AD-associated redistribution of lysosomal hydrolases resulting from changes in CD-MPR expression affects amyloid precursor protein (APP) processing, we stably transfected APP-overexpressing murine L cells with human CD-MPR. As controls for these cells, we also expressed CD-MPR trafficking-mutants that either localize to the plasma membrane (CD-MPRpm) or to early endosomes (CD-MPRendo). Expression of CD-MPR resulted in a partial redistribution of a representative lysosomal hydrolase, cathepsin D, to early endosomal compartments. Turnover of APP and secretion of sAPPα and sAPPβ were not altered by overexpression of any of the CD-MPR constructs. However, secretion of both human Aβ40 and Aβ42 into the growth media nearly tripled in CD-MPR and CD-MPRendo expressing cells when compared to parental or CD-MPRpm expressing cells. Comparable increases were confirmed for endogenous mouse Aβ40 in L cells expressing these CD-MPR constructs but not overexpressing human APP. These data suggest that redistribution of lysosomal hydrolases to early endocytic compartments mediated by increased expression of the CD-MPR may represent a potentially pathogenic mechanism for accelerating Aβ generation in sporadic AD, where the mechanism of amyloidogenesis is unknown.

3.253 Paxillin associates with poly(A)-binding protein 1 at the dense endoplasmic reticulum and the leading edge of migrating cells

Woods, A.J. et al

J. Biol. Chem., ***277(8)***, *6428-6437 (2002)*

Using mass spectrometry we have identified proteins which co-immunoprecipitate with paxillin, an adaptor protein implicated in the integrin-mediated signaling pathways of cell motility. A major component of paxillin immunoprecipitates was poly(A)-binding protein 1, a 70 kDa mRNA-binding protein. Poly(A)-binding protein 1 associated with both the α and β isoforms of paxillin, and this was unaffected by RNase treatment consistent with a protein-protein interaction. The NH_2-terminal region of paxillin (residues 54-313) associated directly with poly(A)-binding protein 1 in cell lysates, and with His-poly(A)-binding protein 1 immobilized in microtiter wells. Binding was specific, saturable and of high affinity (K_d of ≈ 10nM). Cell fractionation studies showed that at steady state, the bulk of paxillin and poly(A)-binding protein 1 was present in the "dense" polyribosome-associated endoplasmic reticulum. However, inhibition of nuclear export with leptomycin B caused paxillin and poly(A)-binding protein1 to accumulate in the nucleus, indicating that they shuttle between the nuclear and cytoplasmic compartments. When cells migrate, poly(A)-binding protein 1 colocalized with paxillinβ at the tips of lamellipodia. Our results suggest a new mechanism whereby a paxillin-poly(A)-binding protein 1 complex facilitates transport of mRNA from the nucleus to sites of protein synthesis at the endoplasmic reticulum and the leading lamella during cell migration.

3.254 Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription

Kegel, K. et al

J. Biol. Chem., ***277(9)***, *7466-7476 (2002)*

Huntingtin is a protein of unknown function that contains a polyglutamine tract, which is expanded in patients with Huntington's disease (HD). We investigated the localization and a potential function for huntingtin in the nucleus. In human fibroblasts from normal and HD patients, huntingtin localized diffusely in the nucleus and in subnuclear compartments identified as speckles, promyelocytic leukemia protein bodies, and nucleoli. Huntingtin-positive nuclear bodies redistributed after treatment with sodium butyrate. By Western blot, purified nuclei had low levels of full-length huntingtin compared with the cytoplasm but contained high levels of N- and C-terminal huntingtin fragments, which tightly bound the nuclear matrix. Full-length huntingtin co-immunoprecipitated with the transcriptional corepressor C-terminal binding protein, and polyglutamine expansion in huntingtin reduced this interaction. Full-length wild-type and mutant huntingtin repressed transcription when targeted to DNA. Truncated N-terminal mutant huntingtin repressed transcription, whereas the corresponding wild-type fragment did not repress transcription. We speculate that wild-type huntingtin may function in the nuclei in the assembly of nuclear matrix-bound protein complexes involved with transcriptional repression and RNA processing. Proteolysis of mutant huntingtin may alter nuclear functions by disrupting protein complexes and inappropriately repressing transcription in HD.

3.255 ORP2, a homolog of oxysterol binding protein, regulates cellular cholesterol metabolism
Laitinen, S. et al
J. Lipid Res., ***43****, 245-255 (2002)*

Oxysterol binding protein (OSBP) related proteins (ORPs) constitute a family that has at least 12 members in humans. In the present study we characterize one of the novel OSBP homologs, ORP2, which we show to be expressed ubiquitously in mammalian tissues. The ORP2 cDNA encodes a deduced 55 kDa protein that lacks a pleckstrin homology (PH) domain, a feature found in the other family members. Sucrose gradient centrifugation analysis of Chinese hamster ovary (CHO) cell post-nuclear supernatant demonstrated that ORP2 is distributed in soluble and membrane-bound fractions. Immunofluorescence microscopy of the endogenous and overexpressed ORP2 in CHO cells suggested that the membrane-bound fraction of the protein localizes to the Golgi apparatus. Stably transfected CHO cells that overexpress ORP2 showed an increase in [^{14}C]cholesterol efflux to serum, apolipoprotein A-I (apoA-I), and phosphatidyl choline vesicles. The proportion of cellular [^{14}C]cholesterol that is esterified and the ACAT activity measured as [^{14}C]oleyl-CoA conversion into cholesteryl [^{14}C]oleate by the cellular membranes, were markedly decreased in the ORP2 expressing cells. Transient high level overexpression of ORP2 interfered with the clearance of a secretory pathway protein marker from the Golgi complex. The results implicate ORP2 as a novel regulator of cellular sterol homeostasis and intracellular membrane trafficking.

3.256 ARL2 and BART enter mitochondria and bind the adenine nucleotide transporter
Sharer, J.D., Shern, J.S., Van Valkenburg, H., Wallace, D.C., and Kahn, R.A.
Mol. Biol. Cell, ***13****, 71-83 (2002)*

The ADP-ribosylation factor-like 2 (ARL2) GTPase and its binding partner binder of ARL2 (BART) are ubiquitously expressed in rodent and human tissues and are most abundant in brain. Both ARL2 and BART are predominantly cytosolic, but a pool of each was found associated with mitochondria in a protease-resistant form. ARL2 was found to lack covalent N-myristoylation, present on all other members of the ARF family, thereby preserving the N-terminal amphipathic α-helix as a potential mitochondrial import sequence. An overlay assay was developed to identify binding partners for the BART-ARL2-GTP complex and revealed a specific interaction with a protein in bovine brain mitochondria. Purification and partial microsequencing identified the protein as an adenine nucleotide transporter (ANT). The overlay assay was performed on mitochondria isolated from five different tissues from either wild-type or transgenic mice deleted for ANT1. Results confirmed that ANT1 is the predominant binding partner for the BART-ARL2-GTP complex and that the structurally homologous ANT2 protein does not bind the complex. Cardiac and skeletal muscle mitochondria from *ant1*$^{-}$*/ant1*$^{-}$ mice had increased levels of ARL2, relative to that seen in mitochondria from wild-type animals. We conclude that the amount of ARL2 in mitochondria is subject to regulation via an ANT1-sensitive pathway in muscle tissues.

3.257 Acidocalcisomes are functionally linked to the contractile vacuole of *Dictyostelium discoideum*
Marchesini, N., Ruiz, F.A., Vieira, M. and Docampo, R.
J. Biol. Chem., ***277(10)****, 8146-8153 (2002)*

The mass-dense granules of *Dictyostelium discoideum* were shown to contain large amounts of phosphorus, magnesium, and calcium, as determined by x-ray microanalysis, either *in situ* or when purified using **iodixanol** gradient centrifugation. The high phosphorus content was due to the presence of pyrophosphate and polyphosphate, which were also present in the contractile vacuoles. Both organelles also possessed a vacuolar H^+ ATPase, an H^+-pyrophosphatase, and a Ca^{2+}-ATPase, as determined by biochemical methods or by immunofluorescence microscopy. The H^+-pyrophosphatase activity of isolated mass-dense granules was stimulated by potassium ions and inhibited by the pyrophosphate analogs aminomethylene-diphosphonate and imidodiphosphate and by KF and N-ethylmaleimide in a dose-dependent manner. The mass-dense granules and the contractile vacuole appeared to contact each other when the cells were submitted to hyposmotic stress. Acetazolamide inhibited the carbonic anhydrase activity of the contractile vacuoles and prolonged their contraction cycle in a dose-dependent manner. Similar effects were observed with the anion exchanger inhibitor 4,4'-diisothiocyanatodihydrostilbene-2, 2'-disulfonic acid and the vacuolar H^+-ATPase inhibitor bafilomycin A_1. Together, these results suggest that the mass-dense granules of *D. discoideum* are homologous to the acidocalcisomes described in protozoan parasites and are linked to the function of the contractile vacuole.

3.258 Agonist-induced translocation of the kinin B_1 receptor to caveolae-related rafts

Sabourin, T., Bastien, L., Bachvarov, D.R., and Marceau, F.
*Mol. Pharmacol., **61(3),** 546-553 (2002)*

The kallikrein-kinin system, activated during inflammatory conditions and the regulation of specific cardiovascular and renal functions, includes two G protein coupled receptors for bradykinin (BK)-related peptides. The B_1 receptor (B_1R) subtype is not believed to undergo agonist-induced phosphorylation and endocytosis. A conjugate made of the rabbit B_1R fused with the yellow variant of green fluorescent protein (YFP) was expressed in mammalian cells. In COS-1 or human embryonic kidney (HEK) 293 cells, the construction exhibited a nanomolar affinity for the agonist radioligand [^{3}H]Lys-des-Arg^9-BK or the antagonist ligand [^{3}H]Lys-[Leu^8]des-Arg^9-BK and a pharmacological profile virtually identical to that of wild-type B_1R. Lys-des-Arg^9-BK stimulation of HEK 293 cells stably expressing B_1R-YFP but not stimulation of untransfected cells released [^{3}H]arachidonate in a phospholipase A_2 assay. B_1R-YFP was visualized as a continuous labeling of the plasma membranes in stably transfected HEK 293 cells (confocal microscopy). Addition of Lys-des-Arg^9-BK (1-100nM) rapidly concentrated the receptor-associated fluorescence into multiple aggregates that remained associated with the plasma membrane (no significant internalization) and colocalized with caveolin-1. This reaction was slowly reversible upon agonist washing at 37°C and prevented pretreatment with B_1R antagonist. β-Cyclodextrin treatment, which extracts cholesterol from membranes and disrupts caveolae-related rafts, prevented agonist-induced redistribution of B_1R-YFP but not the PLA_2 activation mediated by this receptor. The agonist radioligand copurified with caveolin-1 to a greater extent than the tritiated antagonist in buoyant fractions of HEK 293 cells treated with the ligands. Agonist-induced cellular translocation of the kinin B_1R to caveolae-related rafts without endocytosis is a novel variation on the theme of G-protein-coupled receptor adaptation.

3.259 Conversion of raft associated prion protein to the protease-resistant state requires insertion of PrP-res (PrP^{Sc}) into contiguous membranes

Baron, G.S., Wehrly, K., Dorward, D.W., Chesebro, B., and Caughey, B.
*EMBO J., **21(5)**, 1031-1040 (2002)*

Prion protein (PrP) is usually attached to membranes by a glycosylphosphatidylinositol-anchor that associates with detergent-resistant membranes (DRMs), or rafts. To model the molecular processes that might occur during the initial infection of cells with exogenous transmissible spongiform encephalopathy (TSE) agents, we examined the effect of membrane association on the conversion of the normal protease-sensitive PrP isoform (PrP-sen) to the protease-resistant isoform (PrP-res). A cell-free conversion reaction approximating physiological conditions was used, which contained purified DRMs as a source of PrP-sen and brain microsomes from scrapie-infected mice as a source of PrP-res. Interestingly, DRM-associated PrP-sen was not converted to PrP-res until the PrP-sen was either released from DRMs by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), or the combined membrane fractions were treated with the membrane-fusing agent polyethylene glycol (PEG). PEG-assisted conversion was optimal at pH 6-7, and acid pretreating the DRMs was not sufficient to permit conversion without PI-PLC or PEG, arguing against late endosomes/lysosomes as primary compartments for PrP conversion. These observations raise the possibility that generation of new PrP-res during TSE infection requires (i) removal of PrP-sen from target cells; (ii) an exchange of membranes between cells; or (iii) insertion of incoming Prp-res into the raft domains of recipient cells.

3.260 Heterologous desensitization of EGF receptors and PDGF receptors by sequestration in caveolae
Matveev, S.V. and Smart, E.J.
*Am. J. Physiol Cell Physiol, **282**, C935-C946 (2002)*

Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors have been reported to signal via caveolin-containing membranes called caveolae. In contrast, others report that EGF and PDGF receptors are exclusively associated with caveolin-devoid membranes called rafts. Our subcellular fractionation and coimmunoprecipitation studies demonstrate that, in the absence of ligand, EGF and PDGF receptors are associated with rafts. However, in the presence of ligand, EGF and PDGF receptors transiently associate with caveolae. Surprisingly, pretreatment of cells with EGF prevents PDGF-dependent phosphorylation of PDGF receptors and extracellular signal-regulated kinase (ERK) ½ kinase activation. Furthermore, cells pretreated with PDGF prevent EGF-dependent phosphorylation of EGF receptors and ERK1/2 kinase activation. Radioligand binding studies demonstrate that incubation of cells with EGF and PDGF causes both EGF and PDGF receptors to be reversibly sequestered from the extracellular space. Experiments with methyl-β-cyclodextrin, filipin, and antisense caveolin-1 demonstrate that sequestration of the receptors is dependent on cholesterol and caveolin-1. We conclude that ligand-induced stimulation of EGF or PDGF receptors can cause the heterologous desensitization of the other receptor by sequestration in cholesterol-rich, caveolin-containing membranes or caveolae.

3.261 Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton
Yuan, H.Y., Takeuchi, E., and Salant, D.J.
*Am. J. Physiol., Renal Physiol., **282**, F585-F591 (2002)*

Nephrin is an Ig-like transmembrane protein. It is a major component of the podocytes slit diaphragm and is essential for maintaining normal glomular permeability. CD2-associated protein (CD2AP) is also necessary for normal glomerular permeability and is a putative nephrin adaptor molecule. Here, we document that nephrin and CD2AP are linked to the actin cytoskeleton. As detected by Western blot analysis, nephrin and CD2AP were both insoluble when cell membranes from normal rat glomeruli were extracted with 0.5% Triton X-100 (TX-100) at 4°C in the presence of divalent cations, but they were solubilized when the extraction included potassium iodide (KI) to depolymerize F-actin. In addition, a small fraction of the solubilized nephrin and CD2AP was recovered in the low-density fractions of **OptiPrep** flotation gradients, which indicates that a portion of nephrin, possibly associated with CD2AP, resides in a cholesterol- or sphingolipid-rich region of the plasma membrane. Immunofluorescent staining of unfixed sections of normal rat kidney for nephrin, CD2AP, and F-actin was unaltered by treatment with TX-100 but was greatly diminished by addition of KI. Nephrin staining was slightly reduced by cholesterol depletion with methyl-β-cyclodextrin in the presence of TX-100 but was nearly absent after addition of KI. These results document that nephrin anchors the slit diaphragm to the actin cytoskeleton, possibly by linkage to CD2AP, and that nephrin traverses a relatively cholesterol-poor region of the podocytes plasma membrane. In addition, a small pool of actin-associated nephrin and CD2AP resides in lipid rafts, possibly in the cholesterol-rich apical region of the podocytes-foot processes.

3.262 Functional characterization of Δ^3, Δ^2-enoyl-CoA isomerase from rat liver

Zhang, D. et al

J. Biol. Chem., ***277(11)***, *9127-9132 (2002)*

The degradation of unsaturated fatty acids by β-oxidation involves Δ^3, Δ^2-enoyl-CoA isomerase (enoyl-CoA isomerases) that catalyze 3-*cis* → 2-*trans* and 3-*trans* → 2-*trans* isomerizations of enoyl-CoAs and the 2,5 → 3,5 isomerization of dienoyl-CoAs. An analysis of rat liver enoyl-CoA isomerases revealed the presence of a monofunctional enoyl-CoA isomerase (ECI) in addition to mitochondrial enoyl-CoA isomerase (MECI) in mitochondria, whereas peroxisomes contain ECI and multifunctional enzyme 1 (MFE1). Thus ECI, which previously had been described as peroxisomal enoyl-CoA isomerase, was found to be present in both peroxisomes and mitochondria. This enzyme seems to be identical with mitochondrial long-chain enoyl-CoA isomerase (Kilponen, J.M., Palosaari, P.M., and Hiltunen, J.K. 1990 Biochem. J., 269, 223-226). All three hepatic enoyl-CoA isomerases have broad chain length specificities but are distinguishable by their preferences for one of the three isomerization reactions. MECI is most active in catalyzing the 3-*cis* → 2-*trans* isomerization; ECI has a preference for the 3-*trans* → 2- *trans* isomerization, and MFEI is the optimal isomerase for the 2,5 → 3,5 isomerization. A functional characterization based on substrate specificities and total enoyl-CoA isomerase activities in rat liver leads to the conclusion that the 3-*cis* → 2-*trans* and 2,5 → 3,5 isomer-izations in mitochondria are catalyzed overwhelmingly by MECI, whereas ECI contributes significantly to the 3-*trans* → 2-*trans* isomerization. In peroxisomes, ECI is predicted to be the dominant enzyme for the 3-*cis* → 2-*trans* and 3-*trans* → 2-*trans* isomerizations of long-chain intermediates, whereas MFE1 is the key enzyme in the 2,5 → 3,5 isomerization.

3.263 Trafficking and cell surface stability of the epithelial Na^+ channel expressed in epithelial Madin-Darby canine kidney cells

Hanwell, D., Ishikawa, T., Saleki, R., and Rotin, D.

J. Biol. Chem., ***277(12)***, *9772-9779 (2002)*

The apically located epithelial Na^+ channel (αβγ-EnaC) plays a key role in the regulation of salt and fluid transport in the kidney and other epithelia, yet its mode of trafficking to the plasma membrane and its cell surface stability in mammalian cells are poorly understood. Because the expression of EnaC in native tissues/cells is very low, we generated epithelial Madin-Darby canine kidney (MDCK) cells stably expressing αβγ-EnaC, where each subunit is tagged differentially at the intracellular C terminus and the β-subunit is also Myc-tagged at the ectodomain ($\alpha_{HA}\beta_{Myc,T7}\gamma_{FLAG}$). EnaC expression in these cells was verified by immunoblotting with antibodies to the tags, and patch clamp analysis has confirmed that the tagged channel is functional. Moreover, using electron microscopy, we demonstrated apical, but not basal, membrane localization of EnaC in these cells. The glycosylation pattern of the intracellular pool of EnaC revealed peptide N-glycosidase F and endoglycosidase H sensitivity. Surprisingly, the cell surface pool of EnaC, analyzed by surface biotinylation, was also core glycosylated and lacked detectable endoglycosidase H-resistant channels. Extraction of the channel from cells in Triton X-100 demonstrated that both intracellular and cell surface pools of EnaC are largely soluble. Moreover, flotation assays to analyze the presence of EnaC in lipid rafts showed that both intracellular and cell surface pools of this channel are not associated with rafts. We have shown previously that the total cellular pool of EnaC is turned over rapidly ($t_{1/2}$ ~ 1-2 h). Using cycloheximide treatment and surface biotinylation we now demonstrate that the cell surface pool of EnaC has a similarly short half time ($t_{1/2}$ ~ 1 h), unlike the long half-life reported recently for the Xenopus A6 cells. Collectively, these results help elucidate key aspects of EnaC trafficking and turnover rates in mammalian kidney epithelial cells.

3.264 Mannosomes: a molluscan intracellular tubular membrane system related to heavy metal stress
Knigge, T. et al
*Comp. Biochem. and Physiol. Part C, **131**, 259-269 (2002)*

Amongst animals, several hydrogen peroxide-generating oxidases are apparently restricted to molluscs. On of these, D-mannitol oxidase, is concentrated in the alimentary system where it is associated with its own subcellular membrane system of unique tubular morphology, most likely presenting a structural modification of the ER. These structures can be purified by subcellular fractionation and have been termed "mannosomes". Little is known about the functions of mannitol oxidase or of mannosomes, but the previously reported molluscicide-induced increase in mannosomes implies their involvement in a general stress reaction. In this study, we examined the effects of heavy metal stress in the terrestrial gastropod *Arion lusitanicus*. The activity of mannitol oxidase and mannosome abundance were monitored, together with metal effects on heat-shock protein level, and these parameters were compared to heavy metal accumulation in the digestive gland. We found that mannitol oxidase is inhibited by heavy metals more than other oxidases. On the other hand, hsp70 levels and mannosomal protein were increased with enhanced heavy metal stress, the latter indicating a probably increase in the number of mannosome organelles. Thus, stress protein (hsp70) and mannosomal protein were positively correlated with heavy metal accumulation, whereas the enzyme activity showed a negative correlation with increasing heavy metal content of the slugs.

3.265 A proton pumping pyrophosphatase in the Golgi apparatus and plasma membrane vesicles of *Trypanosoma cruzi*
Martinez, R. et al
Mol. & Biochem. Parasitol,, 120, 205-213 (2002)

The proton pumping pyrophosphatase (H^+ -PPase) is an enzyme that has been identified in membranes of plant vacuoles, in the Golgi complex of plants and *Chlamydomonas reinhardii*, and more recently in acidocalcisomes of different trypanosomatids and apicomplexan parasites. Immunofluorescence and immunoelectron microscopy studies using antibodies against the plant enzyme also suggested a plasma membrane localization in different stages of *Trypanosoma cruzi*. In this report we provide immunogold electron microscopy evidence of the presence of the H^+ -PPase in the Golgi complex and plasma membrane of epimastogotes of *T. cruzi*. Pyrophosphate promoted acidification of plasma membrane vesicles as determined using acridine orange. This activity was stimulated by K^+ ions, inhibited by the pyrophosphate analogs imidodiphosphate (IDP) and aminoethylenediphosphate (AMDP) by KF, NaF and DCCD, and it had different responses to ions and inhibitors as compared with the activity present in acidocalcisomes. Surface localization of the H^+ -PPase was confirmed by experiments using biotinylation of cell surface proteins and immunoprecipitation with antibodies against H^+ -PPase. Taken together, these results are consistent with the presence of a functional H^+ -PPase in the plasma membrane of these parasites.

3.266 Alternative splicing unmasks dendritic and axonal targeting signals in metabotropic glutamate receptor 1
Francesconi, A. and Duvoisin, R.M.
*J. Neurosci., **22(6)**, 2196-2205 (2002)*

Precise targeting of neurotransmitter receptors to different neuronal compartments is a fundamental step for the establishment and function of synaptic circuitry. Group I metabotropic glutamate receptors, mGluR1 and mGluR5, control glutamatergic neurotransmission by acting both postsynaptically and presynaptically. Four alternative sliced variants of the mGluR1 gene exist, which differ in their signaling properties and subcellular localization. The present study was undertaken to identify the molecular signals responsible for trafficking of these receptors to different neuronal compartments. Here we report that targeting of mGluR1 to dendrites and axons of transfected retina neurons is controlled by alternative splicing. We have identified in the tail of the receptor a tripeptide motif, which is necessary and sufficient to exclude the splice variant mGluR1b from distal dendrites and to drive it to the axon. This motif, which is present in all the mGluR1 receptors, is masked in mGluR1a by a dominant dendritic signal sequence harbored by the extended C-terminal tail of this splice variant. Furthermore, we show that the identified axonal and dendritic targeting signals are also necessary and sufficient to localize mGluR1b and mGluR1a to the apical and basolateral compartments of Madin-Darby canine kidney cells, respectively, consistent with the existence of common trafficking components for polarized targeting in epithelial cells and neurons.

3.267 Segregation of Bad from lipid rafts is implicated in the induction of apoptosis
Ayllon, V., Fleischer, A., Cayla, X., Garcia, A. and Rebollo, A.
*J. Immunol., **168**, 3387-3393 (2002)*

Many molecules relocate subcellularly in cells undergoing apoptosis. Using coimmunoprecipitation experiments we demonstrate that Bad is not associated to 14-3-3 protein, suggesting a new mechanism for the control of the proapoptotic role of Bad. Here we show, by confocal microscopy and cellular fractionation, that Bad is attached to lipid rafts in IL-4-stimulated cells and thymocytes while associated with mitochondria in IL-4-deprived cells. Disruption of lipid rafts by methyl-β-cyclodextrin treatment induces segregation of Bad from rafts, which correlates with apoptosis. Our results suggest that the interaction of Bad with rafts is a dynamic process regulated by IL-4 and involved in the control of apoptosis.

3.268 Expression of myosin VI within the early endocytic pathway in adult and developing proximal tubules
Biemesderfer, D., Mentone, S.A., Mooseker, M. And Hasson, T.
*Am. J. Physiol., Ren. Physiol**., 282(5)**, F785-F794 (2002)*

Myosin VI is a reverse-direction molecular motor implicated in membrane transport events. Because myosin VI is most highly expressed in the kidney, we investigated its renal localization by using high-resolution immunocytochemical and biochemical methods. Indirect immunofluorescence microscopy revealed myosin VI at the base of the brush border in proximal tubule cells. Horseradish peroxidase uptake studies, which labeled endosomes, and double staining for clathrin adapter protein-2 showed that myosin VI was closely associated with the intermicrovillar (IMV) coated-pit region of the brush border. Localization of myosin VI to the IMV region was confirmed at the electron microscopic level by colloidal gold labeling of ultrathin cryosections. In addition, antigen retrieval demonstrated a small but significant pool of myosin VI on the microvilli. To confirm the association of myosin VI with the IMV compartment, these membranes were separated from other membrane compartments by using 15-25% **OptiPrep** density gradients. Immunoblotting of the gradient fractions confirmed that myosin VI was enriched with markers for the IMV microdomain of the brush border, suggesting that myosin VI associates with proteins in this compartment. Finally, we examined the expression of myosin VI during nephron development. We found myosin VI present in a diffuse cytoplasmic pattern at stage II (S-shaped body phase) and that it was only redistributed fully to the brush border in the stage IV nephron. These studies support a model for myosin VI function in the endocytic process of the proximal tubule.

3.269 Rafts promote assembly and atypical targeting of a nonenveloped virus, rotavirus, in Caco-2 cells
Sapin, C. et al
*J. Virol., **76(9)**, 4591-4602 (2002)*

Rotavirus follows an atypical pathway to the apical membrane of intestinal cells that bypasses the Golgi. The involvement of rafts in this process was explored here. VP4 is the most peripheral protein of the triple-layered structure of this nonenveloped virus. High proportions of VP4 associated with rafts within the cell as early as 3 h postinfection. In the meantime a significant part of VP4 was targeted to the Triton X-100-resistant microdomains of the apical membrane, suggesting that this protein possesses an autonomous signal for its targeting. At a later stage the other structural rotavirus proteins were also found in rafts within the cells together with NSP4, a nonstructural protein required for the final stage of virus assembly. Rafts purified from infected cells were shown to contain infectious particles. Finally purified VP4 and mature virus were shown to interact with cholesterol- and sphingolipid-enriched model lipid membranes that changed their phase preference from inverted hexagonal to lamellar structures. Together these results indicate that a direct interaction of VP4 with rafts promotes assembly and atypical targeting of rotavirus in intestinal cells.

3.270 GCAP1 and rescues rod photoreceptor response in GCAP1/GCAP2 knockout mice
Howes, K.A. et al
The EMBO J., 21(7), 1545-1554 (2002)

Visual transduction in retinal photoreceptors operates through a dynamic interplay of two second messengers, Ca^{2+} and cGMP. Ca^{2+} regulates the activity of guanylate cyclase (GC) and the synthesis of cGMP by acting on a GC-activating protein (GCAP). While this action is critical for rapid termination of the light response, the GCAP responsible has not been identified. To test if GCAP1, one of two GCAPs present in mouse rods, supports the generation of normal flash responses, transgenic mice were generated that express only GCAP1 under the control of the endogenous promoter. Paired flash responses revealed a correlation between the degree of recovery of the rod a-wave and expression levels of GCAP1. In single cell recordings, the majority of the rods generated flash responses that were indistinguishable from wild type. These results demonstrate that GCAP1 at near normal levels supports the generation of wild-type flash responses in the absence of GCAP2.

3.271 Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion
Hu, K. et al
*Nature, **415**, 646-650 (2002)*

Release of neurotransmitter occurs when synaptic vesicles fuse with the plasma membrane. This neuronal exocytosis is triggered by calcium and requires three SNARE (soluble-*N*-ethylmaleimide-sensitive factor attachment protein receptors) proteins: synaptobrevin (also known as VAMP) on the synaptic vesicle, and syntaxin and SNAP-25 on the plasma membrane. Neuronal SNARE proteins form a parallel four-helix bundle that is thought to drive the fusion of opposing membranes. As formation of this SNARE complex in solution does not require calcium, it is not clear what function calcium has in triggering SNARE-mediated membrane fusion. We now demonstrate that whereas syntaxin and SNAP-25 in target membranes are freely available for SNARE complex formation, availability of synaptobrevin on synaptic vesicles is very limited. Calcium at micromolar concentrations triggers SNARE complex formation and fusion between synaptic vesicles and reconstituted target membranes. Although calcium does promote interaction of SNARE proteins between opposing membranes, it does not act by releasing synaptobrevin from synaptic vesicle restriction. Rather, our data suggest a mechanism in which calcium-triggered membrane apposition enables syntaxin and SNAP-25 to engage synaptobrevin, leading to membrane fusion.

3.272 Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation *in vivo*
Walsh, D.M. et al
*Nature, **416**, 535-539 (2002)*

Although extensive data support a central pathogenic role for amyloid β protein (Aβ) in Alzheimer's disease, the amyloid hypothesis remains controversial, in part because a specific neurotoxic species of Aβ and the nature of its effects on synaptic function have not been defined *in vivo*. Here we report that natural oligomers of human Aβ are formed soon after generation of the peptide within specific intracellular vesicles and are subsequently secreted from the cell. Cerebral microinjection of cell medium containing these oligomers and abundant Aβ monomers but no amyloid fibrils markedly inhibited hippocampal long-term potentiation (LTP) in rats *in vivo*. Immunodepletion from the medium of all Aβ species completely abrogated this effect. Pretreatment of the medium with insulin-degrading enzyme, which degrades Aβ monomers but not oligomers, did not prevent the inhibition of LTP. Therefore, Aβ oligomers, in the absence of monomers and amyloid fibrils, disrupted synaptic plasticity *in vivo* at concentrations found in human brain and cerebrospinal fluid. Finally, treatment of cells with γ-secretase inhibitors prevented oligomer formation at doses that allowed appreciable monomer production, and such medium no longer disrupted LTP, indicating that synaptotoxic Aβ oligomers can be targeted therapeutically.

3.273 Polycystin-2 is an intracellular calcium release channel
Koulen, P. et al
*Nature Cell Biol., **4**, 191-197 (2002)*

Polycystin-2, the product of the gene mutated in type 2 autosomal dominant polycystic kidney disease (ADPKD), is the prototypical member of a subfamily of the transient receptor potential (TRP) channel superfamily, which is expressed abundantly in the endoplasmic reticulum (ER) membrane. Here, we show by single channel studies that polycystin-2 behaves as a calcium-activated, high conductance ER channel that is permeable to divalent cations. Epithelial cells overexpressing polycystin-2 show markedly augmented intracellular calcium release signals that are lost after carboxy-terminal truncation or by the introduction of a disease-causing missense mutation. These data suggest that polycystin-2 functions as a calcium –activated intracellular calcium release channel in vivo and that polycystic kidney disease results from the loss of a regulated intracellular calcium release signaling mechanism.

3.274 Specific SHP-2 partitioning in raft domains triggers integrin-mediated signaling via Rho activation
Lacalle, R.A. et al
*J. Cell Biol., **157(2)**, 277-289 (2002)*

Cell signaling does not occur randomly over the cell surface, but is integrated within cholesterol-enriched membrane domains, termed rafts. By targeting SHP-2 to raft domains or to a non-raft plasma membrane fraction, we studied the functional role of rafts in signaling. Serum-depleted, nonattached cells expressing the raft SHP-2 form, but not non-raft SHP-2, display signaling events resembling those observed after fibronectin attachment, such as $ß_1$ integrin clustering, ^{397}Y-FAK phosphorylation, and ERK activation, and also increases Rho-GTP levels. Expression of the dominant negative N19Rho abrogates raft-SHP-2–induced signaling, suggesting that Rho activation is a downstream event in SHP-2 signaling. Expression of a catalytic inactive SHP-2 mutant abrogates the adhesion-induced feedback inhibition of Rho activity, suggesting that SHP-2 contributes to adhesion-induced suppression of Rho activity. Because raft recruitment of SHP-2 occurs physiologically after cell attachment, these results provide a mechanism by which SHP-2 may influence cell adhesion and migration by spatially regulating Rho activity.

3.275 Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus-Merzbacher disease
Simons, M. et al
*J. Cell Biol., **157(2)**, 327-336 (2002)*

Duplications and overexpression of the proteolipid protein (PLP) gene are known to cause the dysmyelinating disorder Pelizaeus-Merzbacher disease (PMD). To understand the cellular response to overexpressed PLP in PMD, we have overexpressed PLP in BHK cells and primary cultures of oligodendrocytes with the Semliki Forest virus expression system. Overexpressed PLP was routed to late endosomes/lysosomes and caused a sequestration of cholesterol in these compartments. Similar results were seen in transgenic mice overexpressing PLP. With time, the endosomal/lysosomal accumulation of cholesterol and PLP led to an increase in the amount of detergent-insoluble cellular cholesterol and PLP. In addition, two fluorescent sphingolipids, BODIPY–lactosylceramide and –galactosylceramide, which under normal conditions are sorted to the Golgi apparatus, were missorted to perinuclear structures. This was also the case for the lipid raft marker glucosylphosphatidylinositol–yellow fluorescence protein, which under normal steady-state conditions is localized on the plasma membrane and to the Golgi complex. Taken together, we show that overexpression of PLP leads to the formation of endosomal/lysosomal accumulations of cholesterol and PLP, accompanied by the mistrafficking of raft components. We propose that these accumulations perturb the process of myelination and impair the viability of oligodendrocytes.

3.276 Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae
Boucher, P. et al
J. Biol. Chem., ***277(18)***, *15507-15513 (2002)*

The low density lipoprotein (LDL) receptor gene family represents a class of multifunctional, endocytic cell surface receptors. Recently, roles in cellular signaling have also emerged. For instance, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein receptor-2 (apoER2) function in a developmental signaling pathway that regulates the lamination of cortical layers in the brain and involves the activation of tyrosine kinases. Furthermore, the cytoplasmic domain of the LDL receptor-related protein (LRP) was found to be a substrate for the non-receptor tyrosine kinase Src, but the physiological significance of this phosphorylation event remained unknown. Here we show that tyrosine phosphorylation of LRP occurs in caveolae and involves the platelet-derived growth factor (PDGF) receptor β and phosphoinositide 3-kinase. Receptor-associated protein, an antagonist of ligand binding to LRP, and apoE-enriched β-VLDL, a ligand for LRP, reduce PDGF-induced tyrosine phosphorylation of the LRP cytoplasmic domain. In the accompanying paper (Loukinova, E., Ranganathan, S., Kuznetsov, S., Gorlatova, N., Migliorini, M., Ulery, P. G., Mikhailenko, I., Lawrence, D. L., and Strickland, D. K. (2002) *J. Biol. Chem.* 277, 15499-15506) Loukinova *et al*. further demonstrate that one form of PDGF, PDGF-BB, binds specifically to LRP and that phosphorylation of LRP requires the activation of Src family kinases. Taken together, these findings provide a biochemical basis for a cellular signaling pathway that involves apoE and LRP.

3.277 EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase
Palmer, A. et al
Mol. Cell, ***9***, *725-737 (2002)*

Ephrins are cell surface-associated ligands for Eph receptors and are important regulators of morphogenic processes such as axon guidance and angiogenesis. Transmembrane ephrinB ligands act as “receptor-like” signaling molecules, in part mediated by tyrosine phosphorylation and by engagement with PDZ domain proteins. However, the underlying cell biology and signaling mechanisms are poorly understood. Here we show that Src family kinases (SFKs) are positive regulators of ephrinB phosphorylation and phosphotyrosine-mediated reverse signaling. EphB receptor engagement of ephrinB causes rapid recruitment of SFKs to ephrinB expression domains and transient SFK activation. With delayed kinetics, ephrinB ligands recruit the cytoplasmic PDZ domain containing protein tyrosine phosphatase PTP-BL and are dephosphorylated. Our data suggest the presence of a switch mechanism that allows a shift from phosphotyrosine/SFK-dependent signaling to PDZ-dependent signaling.

3.278 Induction of protein aggregation in an early secretory compartment by elevation of expression level
Schroder, M., Schafer, R. and Friedl, P.
Biotechnol. Bioeng., ***78(2)***, *131-140 (2002)*

A variety of valuable therapeutic proteins are expressed in mammalian cells. Currently, rate-limiting for secretion of recombinant glycoproteins are activities in the secretory pathway of eukaryotic cells, i.e., folding and glycosylation of the naked polypeptide chain. In this paper we provide evidence that elevation of expression level alone is sufficient to cause intracellular aggregation of a structurally relatively simple glycoprotein, antithrombin III (ATIII). Elevation of expression level by selection for increased drug resistance in Chinese hamster ovary cells stably expressing ATIII resulted in formation of disulfide-bonded aggregates of ATIII. Aggregated ATIII displayed incomplete sialylation and Endo H-sensitivity and located to the endoplasmic reticulum and the cis-Golgi compartment in subcellular fractionations. To explore possible causes for aggregation of ATIII at elevated expression levels we investigated the influence of the two major energy sources of cultured mammalian cells, D-glucose and L-glutamine, on the ATIII-yield. We found that utilization of D-glucose was not limiting for synthesis of ATIII at elevated expression levels. However, the amount of ATIII-synthesized per L-glutamine consumed did not seem to increase steadily with expression level for ATIII, indicating that secretion of ATIII may be limited by the capacity of the cell to utilize L-glutamine.

3.279 Presenilin 1 is required for maturation and cell surface accumulation of nicastrin
Leem, J.Y. et al
J. Biol. Chem., 277(21), 19236-19240 (2002)

Proteolytic processing of amyloid precursor protein generates β-amyloid (Aβ) peptides that are deposited in senile plaques in brains of aged individuals and patients with Alzheimer's disease. Presenilins (PS1 and PS2) facilitate the final step in Aβ production, the intramembranous γ-secretase cleavage of amyloid precursor protein. Biochemical and pharmacological evidence support a catalytic or accessory role for PS1 in γ-sec-retase cleavage, as well as a regulatory role in select membrane protein trafficking. In this report, we demon-strate that PS1 is required for maturation and cell surface accumulation of nicastrin, an integral component of the multimeric γ-secretase complex. Using kinetic labeling studies we show that in *PS1*$^{-/-}$ /*PS2*$^{-/-}$ cells nicastrin fails to reach the medial Golgi compartment, and as a consequence, is incompletely glycosylated. Stable expression of human PS1 restores these deficiencies in *PS1*$^{-/-}$ fibroblasts. Moreover, membrane fractionation studies show co-localization of PS1 fragments with mature nicastrin. These results indicate a novel chaperone-type role for PS1 and PS2 in facilitating nicastrin maturation and transport in the early biosynthetic compartments. Our findings are consistent with PS1 influencing γ-secretase processing at multiple steps, including maturation and intracellular trafficking of substrates and component(s) of the γ-secretase complex.

3.280 Distribution of microsomal triglyceride transfer protein within sub-endoplasmic reticulum in human hepatoma cells
Higashi, Y. et al
*Biochim. Biophys. Acta, **1581**, 127-136 (2002)*

Very low-density lipoprotein (VLDL) particles are formed in the endoplasmic reticulum (ER) through the association of lipids with apolipoprotein B (apoB). Microsomal triglyceride transfer protein (MTP), which transfers lipid molecules to nascent apoB, is essential for VLDL formation in ER. However, little is known of the distribution and interaction of MTP with apoB within ER. In this study, distribution patterns of apoB and MTP large subunits (*l*MTP) within ER were examined. Microsomes prepared from HuH-7 cells, a human hepatoma cell line, were further fractionated into rough ER (RER)-enriched subfractions (ER-I fraction) and smooth ER (SER)-enriched subfractions (ER-II fraction) by **iodixanol** density gradient ultracentrifugation. ApoB was evenly distributed in the ER-I and the ER-II fractions, while 1.5 times more *l*MTP molecules were present in the ER-I fraction than in the ER-II fraction. *l*MTP and apoB were coprecipitated both in the ER-I and in the ER-II fractions by immunoprecipitation whenever anti-apoB or an anti-*l*MTP antibody was used. ApoB-containing lipoprotein particles showed a lower density in the ER-II fraction than those in the ER-I fraction. From these results, it is suggested that MTP can function in both rough and smooth regions of ER in human hepatoma cells.

3.281 Molecular determinants of the sensory and motor neuron-derived factor insertion into plasma membrane

Cabedo, H., Luna, C., Fernandez, A.M., Gallar, J. and Ferrer-Montiel, A.
J. Biol. Chem., ***277(22)***, *19905-19912 (2002)*

The sensory and motor neuron-derived factor (SMDF) is a type III neuregulin that regulates development and proliferation of Schwann cells. Although SMDF has been shown to be a type II protein, the molecular determinants of membrane biogenesis, insertion, and topology remain elusive. Here we used heterologous expression of a yellow fluorescent protein-SMDF fusion protein along with a stepwise deletion strategy to show that the apolar/uncharged segment (Ile^{76}-Val^{100}) acts as an internal, uncleaved membrane insertion signal that defines the topology of the protein. Unexpectedly, removal of the transmembrane segment (TM) did not eliminate completely membrane association of C-terminal fragments. TM-deleted fusion proteins, bearing the amino acid segment (Ser^{283}-Glu^{296}) located downstream to the epidermal growth factor-like motif, strongly interacted with plasma membrane fractions. However, synthetic peptides patterned after this segment did not insert into artificial lipid vesicles, suggesting that membrane interaction of the SMDF C terminus may be the result of a post-translational modification. Subcellular localization studies demonstrated that the 40-kDa form, but not the 83-kDa form, of SMDF was segregated into lipid rafts. Deletion of the N-terminal TM did not affect the interaction of the protein with these lipid microdomains. In contrast, association with membrane rafts was abolished completely by truncation of the protein C terminus. Collectively, these findings are consistent with a topological model for SMDF in which the protein associates with the plasma membrane through both the TM and the C-terminal end domains resembling the topology of other type III neuregulins. The TM defines its characteristic type II membrane topology, whereas the C terminus is a newly recognized anchoring motif that determines its compartmentalization into lipid rafts. The differential localization of the 40- and 83-kDa forms of the neuregulin into rafts and non-raft domains implies a central role in the protein biological activity.

3.282 Amyloid-lowering isocoumarins are not direct inhibitors of γ-secretase

Esler, W.P. et al
Nature Cell Biol., ***4***, *E110 (2002)*

The last step in the production of the amyloid-βprotein (Aβ), the major protein component of the cerebral plaques of Alzheimer's disease, is proteolysis within the transmembrane region of the Amyloid-β Precursor Protein (APP) by γ-secretase. The Notch receptor (N) is processed in a similar manner as part of a signalling mechanism essential for metazoan development. Missense mutations in the polytopic presenilins, PS1 and PS2, cause Alzheimer's disease and alter the specificity of γ-secretase to increase production of a much more aggregation-prone form of Aβ. Knockout studies, site-directed mutagenesis, pharmacological profiling, affinity labelling and biochemical isolation all strongly support the hypothesis that γ-secretase is a complex of integral membrane proteins, an aspartyl protease in which the active site resides between the two subunits of a processed form of PS. As a corollary, PS processes both APP and N.

3.283 Identification, characterization, and localization of a novel kidney polycystin-1-polycystin-2 complex

Newby, L.J. et al
J. Biol. Chem., ***277(23)***, *20763-20773 (2002)*

The functions of the two proteins defective in autosomal dominant polycystic kidney disease, polycystin-1 and polycystin-2, have not been fully clarified, but it has been hypothesized that they may heterodimerize to form a "polycystin complex" involved in cell adhesion. In this paper, we demonstrate for the first time the existence of a native polycystin complex in mouse kidney tubular cells transgenic for *PKD1*, non-transgenic kidney cells, and normal adult human kidney. Polycystin-1 is heavily *N*-glycosylated, and several glycosylated forms of polycystin-1 differing in their sensitivity to endoglycosidase H (Endo H) were found; in contrast, native polycystin-2 was fully Endo H-sensitive. Using highly specific antibodies to both proteins, we show that polycystin-2 associates selectively with two species of full-length polycystin-1, one Endo H-sensitive and the other Endo H-resistant; importantly, the latter could be further enriched in plasma membrane fractions and co-immunoprecipitated with polycystin-2. Finally, a subpopulation of this complex co-localized to the lateral cell borders of *PKD1* transgenic kidney cells. These results demonstrate that polycystin-1 and polycystin-2 interact *in vivo* to form a stable heterodimeric complex and suggest that disruption of this complex is likely to be of primary relevance to the pathogenesis of cyst formation in autosomal dominant polycystic kidney disease.

3.284 Ceramide biosynthesis is required for the formation of the oligomeric H^+ -ATPase Pma1p in the yeast endoplasmic reticulum

Lee, M.C., Hamamoto, S. and Schekman, R.

J. Biol. Chem., ***277(25)***, *23395-23401 (2002)*

The yeast plasma membrane H^+-ATPase Pma1p is one of the most abundant proteins to traverse the secretory pathway. Newly synthesized Pma1p exits the endoplasmic reticulum (ER) via COPII-coated vesicles bound for the Golgi. Unlike most secreted proteins, efficient incorporation of Pma1p into COPII vesicles requires the Sec24p homolog Lst1p, suggesting a unique role for Lst1p in ER export. Vesicles formed with mixed Sec24p-Lst1p coats are larger than those with Sec24p alone. Here, we examined the relationship between Pma1p biosynthesis and the requirement for this novel coat subunit. We show that Pma1p forms a large oligomeric complex of >1 MDa in the ER, which is packaged into COPII vesicles. Furthermore, oligomerization of Pma1p is linked to membrane lipid composition; Pma1p is rendered monomeric in cells depleted of ceramide, suggesting that association with lipid rafts may influence oligomerization. Surprisingly, monomeric Pma1p present in ceramide-deficient membranes can be exported from the ER in COPII vesicles in a reaction that is stimulated by Lst1p. We suggest that Lst1p directly conveys Pma1p into a COPII vesicle and that the larger size of mixed Sec24pLst1p COPII vesicles is not essential to the packaging of large oligomeric complexes.

3.285 The epithelial cell cytoskeleton and intracellular trafficking. I. Shiga toxin B-subunit system: retrograde transport, intracellular vectorization, and more

Ludger, J.

Am. J. Physiol. Gastrointest Liver Physiol., ***283***, *G1-G7 (2002)*

Many intracellular transport routes are still little explored. This is particularly true for retrograde transport between the plasma membrane and the endoplasmic reticulum. Shiga toxin B subunit has become a powerful tool to study this pathway, and recent advances on the molecular mechanisms of transport in the retrograde route and on its physiological function(s) are summarized. Furthermore, it is discussed how the study of retrograde transport of Shiga toxin B subunit allows one to design new methods for the intracellular delivery of therapeutic compounds.

3.286 Hypercholesterolemia promotes a CD36-dependent and endothelial nitric oxide synthase mediated vascular dysfunction

Kincer, J.F. et al

J. Biol. Chem., ***277(26)***, *23525-23533 (2002)*

Numerous studies have implicated either the presence or absence of CD36 in the development of hypertension. In addition, hypercholesterolemia is associated with the loss of nitric oxide-induced vasodilation and the subsequent increase in blood pressure. In the current study, we tested the hypothesis that diet-induced hypercholesterolemia promotes the disruption of agonist-stimulated nitric oxide generation and vasodilation in a CD36-dependent manner. To test this, C57BL/6, apoE null, CD36 null, and apoE/CD36 null mice were maintained on chow or high fat diets. In contrast to apoE null mice fed a chow diet, apoE null mice fed a high fat diet did not respond to acetylcholine with a decrease in blood pressure. Caveolae isolated from *in vivo* vessels did not contain endothelial nitric-oxide synthase and were depleted of cholesterol. Age-matched apoE/CD36 null mice fed a chow or high fat diet responded to acetylcholine with a decrease in blood pressure. The mechanism underlying the vascular dysfunction was reversible because vessels isolated from apoE null high fat-fed mice regained responsiveness to acetylcholine wheri incubated with plasma obtained from chow-fed mice. Further analysis demonstrated that the plasma low density lipoprotein fraction was responsible for depleting caveolae of cholesterol, removing endothelial nitric-oxide synthase from caveolae, and preventing nitric oxide production. In addition, the pharmacological removal of caveola cholesterol with cyclodextrin mimicked the effects caused by the low density lipoprotein fraction. We conclude that the ablation of CD36 prevented the negative impact of hypercholesterolemia on agonist-stimulated nitric oxide-mediated vasodilation in apoE null mice. These studies provide a direct link between CD36 and the early events that underlie hypercholesterolemia-mediated hypertension and mechanistic linkages between CD36 function, nitric-oxide synthase activation, caveolae integrity, and blood pressure regulation.

3.287 Construction of a catalytically inactive cholesterol oxidase mutant: inverstigation of the interplay between active site-residues glutamate 361 and histidine 447.
Yin, Y., Liu, P., Anderson, R.G.W. and Sampson, N.S.
*Arch. Biochem. Biophys., **402**, 235-242 (2002)*

Cholesterol oxidase catalyzes the oxidation of cholesterol to cholest-5-en-3-one and its subsequent isomerization into cholest-4-en-3-one. Two active-site residues, His447 and Glu361, are important for catalyzing the oxidation and isomerization reactions, respectively. Double-mutants were constructed to test the interplay between these residues in catalysis. We observed that the k_{cat} of oxidation for the H447Q/E361Q mutant was 3-fold less than that for H447Q and that the k_{cat} of oxidation for the H447E/E361Q mutant was 10-fold slower than that for H447E. Because both doubles-mutants do not have a carboxylate at position 361, they do not catalyze isomerization of the reaction intermediate cholest-5-en-3-one to cholest-4-en-3-one. These results suggest that Glu361 can compensate for the loss of histidine at position 447 by acting as a general base catalyst for oxidation of cholesterol. Importantly, the construction of the double-mutant H447E/E361Q yields an enzyme that is 31,000-fold slower than wild type in k_{cat} for oxidation. The H447E/E361Q mutant is folded like native enzyme and still associates with model membranes. Thus, this mutant may be used to study the effects of membrane binding in the absence of catalytic activity. It is demonstrated that in assays with caveolae membrane fractions, the wild-type enzyme uncouples platelet-derived growth factor receptor β (PDGFRβ) autophosphorylation from tyrosine phosphorylation of neighboring proteins, and the H447E/E361Q mutant does not. Thus maintenance of membrane structure by cholesterol is important for PDGFRβ-mediated signaling. The cholesterol oxidase mutant probe described will be generally useful for investigating the role of membrane structure in signal transduction pathways in addition to the PDGFRβ-dependent pathway tested.

3.288 Optimization of the workup procedure for the analysis of 8-oxo-7, 8-dihydro-2'-deoxyguanosine with electrochemical detection
Hofer, T. and Moller, L.
*Chem. Res. Toxicol., **15**, 426-432 (2002)*

The artifactual generation of the biomarker for oxidative stress, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), during the workup procedure for its analysis is a difficult problem to solve, and the responsible factors are unclear. Here, peroxide removal and other antioxidant procedures during workup were compared using a limited amount of rat liver (50 mg) **as** starting material, with subsequent hydrolysis of 50 μg of DNA. A cold (0°C) high salt GTC (4 M guanidine thiocyanate) non-phenol DNA extraction method was developed where DNA is quickly isolated. GSH (reduced glutathione) generated artifactual formation of 8-oxodG during the workup procedure, whereas H_20_2 removal using catalase, Fe^{3+} removal and passivation using desferal, peroxide removal using glutathione peroxidase, ebselen and a peroxidase mimic lowered the 8-oxodG levels, all identifying peroxides as the responsible oxidants. Desferal was more protective when excluding Mg^{2+} and Ca^{2+} from buffers but was found to disturb the electrochemical detector when repeatedly injected five to six times, even at 100 μM. Addition of the OH• scavenger ethanol in all steps at 2% v/v had no protective effect. Zn^{2+} was found necessary for efficient DNA hydrolysis using nuclease P_1 which was poor below 37°C. Use of water substitutes was tested but inhibited DNA hydrolysis completely. $H_2{}^{18}O$ could, however, work for mass spectrometry methods. Long-term (38 days) storage of 0.5% v/v Triton X-100 generated more 8-oxodG than Tween 20 when Incubated with free dG. The cold GTC DNA extraction method was used for analysis of freshly isolated human lymphocytes/monocytes from 60 healthy men using catalase and TEMPO as antioxidants, giving a background level of 0.074 ± 0.027 8-oxodG/10^5 dG (or 16 8-oxodG/10^8 nucleotides or 1943 8-oxodG/nuclei) which is probably the lowest value obtained yet. No increase with age was seen. Oxidation of dG to 8-oxodG during workup was found to fit a mathematically defined curve, and a calculated background level of 0.047 8-oxodG/10^5 dG was obtained. To obtain more reliable results it is recommended that control samples are included during the workup procedure, having an equal amount of cells (or DNA) as the exposed samples.

3.289 Studying the behavior of mitochondria

Nunnari, J., Wong, E.D., Meesusen, S. and Wagner, J.A.
Methods in Enzymol., ***351***, *381-393 (2002)*

Yeast mitochondria form dynamic tubular structures that are distributed uniformly at the cell cortex and contain an average of 50 to 100 copies of the mitochondrial genome (mtDNA) packaged into higher order nucleoid structures. *Saccharamyces cerevisiae* is an ideal system for studying the mechanisms of both mitochondrial structure and mtDNA maintenance. Even after cells lose mtDNA, often as a secondary consequence of abnormal mitochondrial morphology, they can still be propagated and studied when a fermentable carbon source such as glucose is present. However, because of its role in various metabolic processes, the mitochondrial organelle, even in the absence of mtDNA, is an essential structure that cannot be created de novo. Therefore, a daughter cell survives only if a portion of the mitochondrion is inherited from the mother cell before cytokinesis..
Much insight into the mechanisms that govern the shape, distribution, and movement of mitochondria, as well as the behavior of mtDNA, has been gained through the use of genetic, cytological, and biochemical approaches. This chapter describes some of the cytological and biochemical techniques that have been developed and used in our laboratory and by others in the field to study the behavior of this complex organelle.

3.290 Lipid microdomains are required sites for the selective endocytosis and nuclear translocation of IFN-γ, its receptor chain IFN-γ receptor-1, and phosphorylation and nuclear translocation of STAT1α

Subramanian, P.S. and Johnson, H.M.
J. Immunol, ***169***, *1959-1969 (2002)*

IFN-γ contains a nuclear localization sequence that may play a role in the nuclear transport of activated STAT1α via a complex of IFN-γ/IFN-γ receptor (IFNGR)-1/STAT1α with the nuclear importer nucleoprotein interactor 1. In this study, we examine the mechanism of endocytosis of IFNGR-1 and the relationship of its nuclear translocation to that of STAT1α. In untreated WISH cells, both IFNGR-1 and IFNGR-2 were constitutively localized within caveolae-like microdomains isolated from plasma membrane. However, treatment of cells with IFN-γ resulted in rapid migration of IFNGR-1, but not IFNGR-2, from these microdomains. Filipin pretreatment, which specifically inhibits endocytosis from caveolae-like microdomains, inhibited the nuclear translocation of IFN-γ and IFNGR-1 as well as the tyrosine phosphorylation and nuclear translocation of STAT1α, but did not affect the binding of IFN-γ to these cells. In the Jurkat T lymphocyte cell line, which does not express caveolin-1, nuclear translocation of IFNGR-1 and STAT1α were similarly inhibited by filipin pretreatment. Isolation of lipid microdomains from Jurkat cells showed that both IFNGR-1 and IFNGR-2 were associated with lipid microdomains only after stimulation with IFN-γ, suggesting that the IFNGR subunits are recruited to lipid microdomains by IFN-γ binding in lymphocytes (Jurkat) in contrast to their constitutive presence in epithelial (WISH) cells. In contrast, treatments that block clathrin-dependent endocytosis did not inhibit either activation or nuclear translocation of STAT1α or the nuclear translocation of IFN- or IFNGR-1. Thus, membrane lipid microdomains play an important role in IFN-γ-initiated endocytic events involving IFNGR-1, and the nuclear translocation of IFN-γ, IFNGR-1, and STAT1α

3.291 Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts

Yamabhai, M. and Anderson, R.G.W.
J. Biol. Chem., ***277(28)***, *24843-24846 (2002)*

Previous studies have shown that ~60% of the epidermal growth factor receptors (EGFRs) in quiescent fibroblasts are concentrated in the caveolae/raft fraction from purified plasma membranes. This high degree of localization suggests the EGFR contains targeting information for lipid domains. We have used mutagenesis to determine that the region of the receptor that controls targeting to caveolae/rafts resides in the juxtamembrane, second cysteine-rich region. A 60-amino acid-long sequence within this region that is continuous with the transmembrane domain was sufficient to target the transmembrane and cytoplasmic tails of both EGFR and the low density lipoprotein receptor to caveolae/rafts. Two *N*-linked sugars in this segment were not required for proper targeting, although unglycosylated wild-type receptors did not localize properly. We conclude that, in contrast to signals for coated pit localization that are in the

cytoplasmic tail, the targeting information for caveolae/rafts is on the extracellular side of the EGFR very close to the membrane.

3.292 Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins
Yang, D-S, et al
J. Biol. Chem., 277(31), 28135-28142 (2002)

Nicastrin is an integral component of the high molecular weight presenilin complexes that control proteolytic processing of the amyloid precursor protein and Notch. We report here that nicastrin is most probably a type 1 transmembrane glycoprotein that is expressed at moderate levels in the brain and in cultured neurons. Immunofluorescence studies demonstrate that nicastrin is localized in the endoplasmic reticulum, Golgi, and a discrete population of vesicles. Glycosidase analyses reveal that endogenous nicastrin undergoes a conventional, trafficking-dependent maturation process. However, when highly expressed in transfected cells, there is a disproportionate accumulation of the endo-β-*N*-acetylglucosaminidase H-sensitive, immature form, with no significant increase in the levels of the fully mature species. Immunoprecipitation revealed that presenilin-1 interacts preferentially with mature nicastrin, suggesting that correct trafficking and co-localization of the presenilin complex components are essential for activity. These findings demonstrate that trafficking and post-translational modifications of nicastrin are tightly regulated processes that accompany the assembly of the active presenilin complexes that execute -secretase cleavage. These results also underscore the caveat that simple overexpression of nicastrin in transfected cells may result in the accumulation of large amounts of the immature protein, which is apparently unable to assemble into the active complexes capable of processing amyloid precursor protein and Notch.

3.293 Blocking of HIV-1 infection by targeting CD4 to nonraft membrane domains
del Real, G. et al
J. Exp. Med., 196(3), 293-301 (2002)

Human immunodeficiency virus (HIV)-1 infection depends on multiple lateral interactions between the viral envelope and host cell receptors. Previous studies have suggested that these interactions are possible because HIV-1 receptors CD4, CXCR4, and CCR5 partition in cholesterol-enriched membrane raft domains. We generated CD4 partitioning mutants by substituting or deleting CD4 transmembrane and cytoplasmic domains and the CD4 ectodomain was unaltered. We report that all CD4 mutants that retain raft partitioning mediate HIV-1 entry and CD4-induced Lck activation independently of their transmembrane and cytoplasmic domains. Conversely, CD4 ectodomain targeting to a nonraft membrane fraction results in a CD4 receptor with severely diminished capacity to mediate Lck activation or HIV-1 entry, although this mutant binds gp120 as well as CD4wt. In addition, the nonraft CD4 mutant inhibits HIV-1 X4 and R5 entry in a $CD4^+$ cell line. These results not only indicate that HIV-1 exploits host membrane raft domains as cell entry sites, but also suggest new strategies for preventing HIV-1 infection.

3.294 Stomatin-related olfactory protein, SRO, spedifically expressed in the murine olfactory sensory neurons
Kobayakawa, K. et al
*J. Neurosci., **22(14)**, 5931-5937 (2002)*

We identified a stomatin-related olfactory protein (SRO) that is specifically expressed in olfactory sensory neurons (OSNs). The mouse *sro* gene encodes a polypeptide of 287 amino acids with a calculated molecular weight of 32 kDa. SRO shares 82% sequence similarity with the murine stomatin, 78% with *Caenorhabditis elegans* MEC-2, and 77% with *C. elegans* UNC-1. Unlike other stomatin-family genes, the *sro* transcript was present only in OSNs of the main olfactory epithelium. No *sro* expression was seen in vomeronasal neurons. SRO was abundant in most apical dendrites of OSNs, including olfactory cilia. Immunoprecipitation revealed that SRO associates with adenylyl cyclase type III and caveolin-1 in the low-density membrane fraction of olfactory cilia. Furthermore, anti-SRO antibodies stimulated cAMP production in fractionated cilia membrane. SRO may play a crucial role in modulating odorant signals in the lipid rafts of olfactory cilia.

3.295 Differential sorting and fate of endocytosedGPI-anchored proteins
Fivaz, M. et al
*EMBO J., **(21/15)**, 3989-4000 (2002)*

In this paper, we studied the fate of endocytosed glycosylphosphatidyl inositol anchored proteins (GPI-APs) in mammalian cells, using aerolysin, a bacterial toxin that binds to the GPI anchor, as a probe. We find that GPI-APs are transported down the endocytic pathway to reducing late endosomes in BHK cells, using biochemical, morphological and functional approaches. We also find that this transport correlates with the association to raft-like membranes and thus that lipid rafts are present in late endosomes (in addition to the Golgi and the plasma membrane). In marked contrast, endocytosed GPI-APs reach the recycling endosome in CHO cells and this transport correlates with a decreased raft association. GPI-APs are, however, diverted from the recycling endosome and routed to late endosomes in CHO cells, when their raft association is increased by clustering seven or less GPI-APs with an aerolysin mutant. We conclude that the different endocytic routes followed by GPI-APs in different cell types depend on the residence time of GPI-APs in lipid rafts, and hence that raft partitioning regulates GPI-APs sorting in the endocytic pathway.

3.296 Akt1 regulates a JNK scaffold during excitotoxic apoptosis
Kim, A.H. et al
*Neuron, **35**, 697-709 (2002)*

Cell survival is determined by a balance among signaling cascades, including those that recruit the Akt and JNK pathways. Here we describe a novel interaction between Akt1 and JNK interacting protein 1 (JIP1), a JNK pathway scaffold. Direct association between Akt1 and JIP1 was observed in primary neurons. Neuronal exposure to an excitotoxic stimulus decreased the Akt1-JIP1 interaction and concomitantly increased association between JIP1 and JNK. Akt1 interaction with JIP1 inhibited JIP1-mediated potentiation of JNK activity by decreasing JIP1 binding to specific JNK pathway kinases. Consistent with this view, neurons from Akt1-deficient mice exhibited higher susceptibility to kainate than wild-type littermates. Overexpression of Akt1 mutants that bind JIP1 reduced excitotoxic apoptosis. These results suggest that Akt1 binding to JIP1 acts as a regulatory gate preventing JNK activation, which is released under conditions of excitotoxic injury.

3.297 Hsp90 interactions and acylation target the G protein $G\alpha_{12}$ but not $G\alpha_{13}$ to lipid rafts
Waheed, A.A. and Jones, T.L.Z.,
*J. Biol. Chem., **277(36)**, 32409-32412 (2002)*

The heterotrimeric G proteins, G_{12} and G_{13}, are closely related in their sequences, signaling partners, and cellular effects such as oncogenic transformation and cytoskeletal reorganization. Yet G_{12} and G_{13} can act through different pathways, bind different proteins, and show opposing actions on some effectors. We investigated the compartmentalization of G_{12} and G_{13} at the membrane because other G proteins reside in lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids. Lipid rafts were isolated after cold, nonionic detergent extraction of cells and gradient centrifugation. $G\alpha_{12}$ was in the lipid raft fractions, whereas $G\alpha_{13}$ was not associated with lipid rafts. Mutation of Cys-11 on $G\alpha_{12}$, which prevents its palmitoylation, partially shifted $G\alpha_{12}$ from the lipid rafts. Geldanamycin treatment, which specifically inhibits Hsp90, caused a partial loss of wild-type $G\alpha_{12}$ and a complete loss of the Cys-11 mutant from the lipid rafts and the appearance of a higher molecular weight form of $G\alpha_{12}$ in the soluble fractions. These results indicate that acylation and Hsp90 interactions localized $G\alpha_{12}$ to lipid rafts. Hsp90 may act as both a scaffold and chaperone to maintain a functional $G\alpha_{12}$ only in discrete membrane domains and thereby explain some of the nonoverlapping functions of $G\alpha_{12}$ and $G\alpha_{13}$ and control of these potent cell regulators.

3.298 Complex N-linked glycosylated nicastrin associates with active γ-secretase and undergoes tight cellular regulation

Taylor Kimberly, W. et al

J. Biol. Chem., 277(38), 35113-35117 (2002)

The intramembranous proteolysis of Notch and the amyloid precursor protein by γ-secretase exemplifies an unusual and newly recognized mechanism of signal transduction in multicellular organisms. Here, we show that only a form of nicastrin (NCT) containing *N*-linked complex oligosaccharides is present in active γ-secretase complexes. Overexpression of NCT does not generate more of this mature protein, a phenomenon analogous to the strictly regulated formation of mature presenilin heterodimers from immature holoprotein. The absence of presenilin severely limits the maturation of NCT, yet combined overexpression of both proteins does not increase respective mature types. Taken together, our findings describe unusual regulatory features of this key signaling protease: the association of NCT with γ-secretase is tightly regulated via glycosylation; at least one other cofactor exists; the least abundant member of the complex becomes limiting; and the cofactor that serves this role may vary by cell type.

3.299 FATS1 channels exogenous FA into 1,2,3-triacyl-sn-glycerol and down-regulates sphingomyelin and cholesterol metabolism in growing 293 cells

Hatch, G.M., Smith, A.J., Xu, F.Y., Hall, A.M. and Bernlohr, D.A.

*J. Lipid. Res., **43**, 1380-1389 (2002)*

Biosynthesis of lipids was investigated in growing 293 cells stably expressing fatty acid (FA) transport protein 1 (FATP1), a bifunctional polypeptide with FA transport as well as fatty acyl-CoA synthetase activity. In short-term (30 s) incubations, FA uptake was increased in FATP1 expressing cells (C8 cells) compared with the vector (as determined by BODIPY 3823 staining and radioactive FA uptake). In long-term (4 h) incubations, incorporation of [^{14}C]acetate, [3H]oleic acid, or [^{14}C]lignoceric acid into 1,2,3-triacyl-*sn*-glycerol (TG) was elevated in C8 cells compared with vector, whereas incorporation of radiolabel into glycerophospholipids was unaltered. The increase in TG biosynthesis correlated with an increase in 1,2-diacyl-*sn*-glycerol acyltransferase activity in C8 cells compared with vector. In contrast, incorporation of [^{14}C]acetate into sphingomyelin (SM) and cholesterol, and [3H]oleic acid or [^{14}C]lignoceric acid into SM was reduced due to a reduction in de novo biosynthesis of these lipids in C8 cells compared with vector. The results indicate that exogenously supplied FAs, and their subsequently produced acyl-CoAs, are preferentially channeled by an FATP1 linked mechanism into the TG biosynthetic pathway and that such internalized lipids down-regulate de novo SM and cholesterol metabolism in actively growing 293 cells.

3.300 Inversin forms a complex with catenins and N-cadherin in polarized epithelial cells

Nürnberger, J., Bacallao, R.L. and Phillips, C.P.

*Mol. Biol. Cell, **13**, 3096-3106 (2002)*

Nephrogenesis starts with the reciprocal induction of two embryonically distinct analages, metanephric mesenchyme and ureteric bud. This complex process requires the refined and coordinated expression of numerous developmental genes, such as *inv*. Mice that are homozygous for a mutation in the *inv* gene (*inv/inv*) develop renal cysts resembling autosomal-recessive polycystic kidney disease. The gene locus containing *inv* has been proposed to serve as a common modifier for some human and rodent polycystic kidney disease phenotypes. We generated polyclonal antibodies to inversin to study its subcellular distribution, potential binding partners, and functional aspects in cultured murine proximal tubule cells. A 125-kDa inversin protein isoform was found at cell-cell junctions. Two inversin isoforms, 140- and 90-kDa, were identified in the nuclear and perinuclear compartments. Plasma membrane allocation of inversin is dependent upon cell-cell contacts and was redistributed when cell adhesion was disrupted after incubation of the cell monolayer with low-calcium/EGTA medium. We further show that the membrane-associated 125-kDa inversin forms a complex with N-cadherin and the catenins. The 90-kDa nuclear inversin complexes with β-catenin. These findings indicate that the *inv* gene product functions in several cellular compartments, including the nucleus and cell-cell adhesion sites.

3.301 Differential localization of the vacuolar H^+ pump with G subunit isoforms (G1 and G2) in mouse neurons

Murata, Y. et al
J. Biol. Chem., ***277(39)***, *36296-36303 (2002)*

Vacuolar H^+-ATPases (V-ATPases), a family of multimeric proton pumps, are involved in a wide variety of physiological processes. We have identified two mouse genes, *Atp6g1* and *Atp6g2*, encoding the *G*1 and *G*2 isoforms of the V-ATPase *G* subunit, respectively. *G*1 was distributed ubiquitously in the tissues examined, whereas *G*2 was specifically distributed in central nervous system neurons. *G*1 was expressed at an early embryonic stage, whereas *G*2 transcription was significantly induced at 10.5 days postcoitus (embryonic day 10.5, *i.e.* 2 days before axon outgrowth). Both *G*1 and *G*2 were strongly expressed in cortical and hippocampal neurons, cerebellar granule cells, and Purkinje cells. Immunohistochemistry with isoform-specific antibodies revealed that *G*2 was localized in cell bodies, dendrites, and axons. In addition, electron microscopy and subcellular fractionation indicated that *G*2 was localized in synaptic vesicles, whereas *G*1 was not detectable. *G*1 and *G*2 exhibit 62% identity, and both isoforms were immunoprecipitated with the *c* and *A* subunits of V-ATPase. *G*2 could complement the yeast deletion mutant Δ*vma10*, which lacks the *G* subunit. The V-ATPases containing the *G*1 and *G*2 isoforms, respectively, showed similar $K_{m(ATP)}$ values and maximal velocity. These results indicate that *G*1 and *G*2 are *bona fide* subunits of V-ATPases and that the enzyme with the *G*2 isoform is involved in synaptic vesicle acidification.

3.302 Myristoylation as a target for inhibiting HIV assembly: Unsaturated fatty acids block viral budding

Lindwasser, O.W. and Resh, M.D.
Proc. Natl. Acad. Sci.,99(20), 13037-13042 (2002)

Modification of HIV-1 Gag with myristic acid, a saturated 14-carbon fatty acid (14:0), is essential for HIV-1 assembly. We recently showed that exogenous treatment of cells with unsaturated 14-carbon fatty acids, 5-*cis*-tetradecenoic acid (14:1*n*-9) and 5-*cis*,8-*cis*-tetradecadienoic acid (14:2*n*-6), reduces the affinity of some myristoylated proteins for plasma membrane rafts, membrane subdomains that have been shown to be required for efficient assembly of HIV. Here we demonstrate that treatment of cells with 14:1*n*-9 and 14:2*n*-6 fatty acids reduced the affinity of Gag for rafts but not membranes in general. Furthermore, treatment of cells with 14-carbon unsaturated fatty acids inhibited Gag-driven particle assembly. These effects most likely reflect covalent modification of Gag with unsaturated fatty acids. Treatment with 14:1*n*-9 and 14:2*n*-6 fatty acids did not alter intracellular protein trafficking, nor did it reduce cell viability. These studies suggest a strategy to attack HIV assembly by selectively altering the patterns of fatty acid modification.

3.303 Elevation of cytochrome P450-immunopositive protein and DNA damage in mussels (*Mytilus edulis*) transplanted to a contaminated site

Shaw, J.P., Large, A.T., Livingstone, D.R., Doyotte, A., Renger, J., Chipman, J.K. and Peters, L.D.
Marine Environ. Res., **54**, 505-509 (2002)

Mytilus edulis were collected from a reference site (Port Quin) and an urban/industrial contaminated site (New Brighton) in the UK during June 1999.Levels of PCBs (Σ7 congeners)
and CB-138 were determined to be, respectively, 21 fold and 16 fold higher in the mussel digestive glands from New Brighton. Levels of CYP1A-immunopositive protein were 1.5 fold higher (P<0.05) at the polluted site but the levels of DNA strand breaks were 1.3 fold higher (P<0.05) at the reference site. Mussels from Port Quin were placed in cages at both sites and both transplanted and indigenous populations sampled in September (13 weeks). Mussels transplanted from the reference site to the industrial site, reported elevated levels of CYP1A-immunopositive protein (1.4 fold; P<0.05) and higher levels of DNA damage (1.2 fold; P<0.05) compared to caged populations at the reference site and a PCB loading similar to the populations from the polluted site.Moreover, transplanted mussels had DNA damage 1.8 fold greater (P<0.05) than indigenous mussels at the transplant site. These changes were small but significant when compared to the observed temporal changes in the indigenous populations.

3.304 Rat liver acyl-CoA synthetase 4 is a peripheral-membrane protein located in two distinct subcellular organelles, peroxisomes and mitochondrial-associated membranes
Lewin T.M., Van Horn, C.G., Krisans, S.K. and Coelman, R.A.
Arch. Biochem. Biophys., **404**, 263-270 (2002)

Obesity and non-insulin-dependent diabetes favor storage of fatty acids in triacylglycerol over oxidation. Recently, individual acyl-CoA synthetase (ACS) isoforms have been implicated in the channeling of fatty acids either toward lipid synthesis or toward oxidation. Although ACS1 had been localized to three different subcellular regions in rat liver, endoplasmic reticulum, mitochondria, and peroxisomes, the study had used an antibody raised against the full-length ACS1 protein which cross-reacts with other isoforms, probably because all ACS family members contain highly conserved amino acid sequences. Therefore, we examined the subcellular location of ACS1, ACS4, and ACS5 in rat liver to determine which isoform was present in peroxisomes, whether the ACSs were intrinsic membrane proteins, and which ACS isoforms were up-regulated by PPARα ligands. Non-cross-reacting ACS1, ACS4, and ACS5 peptide antibodies showed that ACS4 was the only ACS isoform present in peroxisomes isolated from livers of gemfibrozil-treated rats. ACS4 was also present in fractions identified as mitochondria associated membrane (MAM). ACS1 was present in endoplasmic reticulum fractions and ACS5 was present in mitochondrial fractions. Incubation with troglitazone, a specific inhibitor of ACS4, decreased ACS activity in the MAM fractions 30–45% and in the peroxisomal fractions about 30%. Because the signal for ACS4 protein in peroxisomes was so strong compared to the MAM fraction, we examined ACS4 mRNA abundance in livers of rats treated with the PPARα agonist GW9578. Treatment with GW9578 increased ACS4 mRNA abundance 40% and ACS1 mRNA 25%. Although we had originally proposed thatACS4 is linked to triacylglycerol synthesis, it now appears that ACS4 may also be important in activating fatty acids destined for peroxisomal oxidation. We also determined that, unlike ACS1 and 5, ACS4 is not an intrinsic membrane protein. This suggests that ACS4 is probably targeted and linked to MAM and peroxisomes by interactions with other proteins.

3.305 Multicompartmental distribution of tuberous sclerosis gene products, hamartin and tuberin
Yamamoto, Y., Jones, K.A., Mak, B.C., Muehlenbachs, A. and Yeung, R.S.
Arch. Biochem. Biophys., **404**, 210-217 (2002)

Mutations of the TSC1 and TSC2 genes give rise to the clinical disorder of tuberous sclerosis characterized by the development of hamartomas predominantly affecting the central nervous system, kidney, skin, lung, and heart. The function of the gene products, hamartin and tuberin, is not well understood but we have previously suggested a role in vesicular transport. To define the subcellular compartment(s) involved with these two proteins, biochemical characterization of hamartin and tuberin was performed in primary tissues and cell lines. Fractionation of cell lysates identifed both proteins in the cytosolic, microsomal, and cytoskel-etal compartments. In each of these fractions, hamartin and tuberin formed a stable complex in coimmuno-precipitation analyses. Further, they colocalized extensively in discrete, vesicular structures in the cyto-plasm. Within the microsomal compartment, hamartin and tuberin behaved as peripheral membrane proteins that associate with the cytosolic leaflet of membranous domains. Immunoisolation of tuberin-bound vesicles using magnetic beads showed an enrichment of rap1, rab5, and caveolin-1, all of which have been found in specialized lipid microdomains, caveolae. Our data suggest that hamartin and tuberin are multicompart-mental proteins that partially reside in caveolin-1-enriched structures and potentially affect their signaling.

3.306 Sphingolipid and cholesterol dependence of alphavirus membrane fusion
Waarts, B-L., Bittman, R. and Wilschutt, J.
J. Biol. Chem., ***277(41)***, *38141-38147 (2002)*

Semliki Forest virus (SFV) and Sindbis virus (SIN) are enveloped viruses that infect their host cells by receptor-mediated endocytosis and subsequent fusion from within acidic endosomes. Fusion of the viral envelope requires the presence of both cholesterol and sphingolipids in the target membrane. This is suggestive of a possible involvement of sphingolipid-cholesterol microdomains, or "lipid rafts," in the membrane fusion and cell entry process of the virus. In this study, large unilamellar vesicles (LUVs) were prepared from synthetic sphingolipids and sterols that vary with respect to their capacity to promote microdomain formation, as assessed by gradient flotation analysis in the presence of Triton X-100. SFV and SIN fused with LUVs irrespective of the presence or absence of Triton X-100-insoluble microdomains. These results suggest that SFV and SIN do not require the presence of lipid rafts for fusion with target

membranes. Furthermore, it is not necessary for sphingolipids to reside in a detergent-insoluble complex with cholesterol to promote SFV or SIN fusion.

3.307 ER-X: A novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury
Toran-Allerand, C.D. et al
J. Neurosci., 22(19), 8391-8401 (2002)

We showed previously in neocortical explants, derived from developing wild-type and estrogen receptor (ER)-α gene-disrupted (ERKO) mice, that both 17α- and 17β-estradiol elicit the rapid and sustained phosphorylation and activation of the mitogen-activated protein kinase (MAPK) isoforms, the extracellular signal-regulated kinases ERK1 and ERK2. We proposed that the ER mediating activation of the MAPK cascade, a signaling pathway important for cell division, neuronal differentiation, and neuronal survival in the developing brain, is neither ER-α nor ER-β but a novel, plasma membrane-associated, putative ER with unique properties. The data presented here provide further evidence that points strongly to the existence of a high-affinity, saturable, ^{3}H-estradiol binding site (K_d, ~1.6 nM) in the plasma membrane. Unlike neocortical ER-α, which is intranuclear and developmentally regulated, and neocortical ER-β, which is intranuclear and expressed throughout life, this functional, plasma membrane-associated ER, which we have designated "ER-X," is enriched in caveolar-like microdomains (CLMs) of postnatal, but not adult, wild-type and ERKO neocortical and uterine plasma membranes. We show further that ER-X is functionally distinct from ER-α and ER-β, and that, like ER-α, it is re-expressed in the adult brain, after ischemic stroke injury. We also confirmed in a cell-free system that ER-α is an inhibitory regulator of ERK activation, as we showed previously in neocortical cultures. Association with CLM complexes positions ER-X uniquely to interact rapidly with kinases of the MAPK cascade and other signaling pathways, providing a novel mechanism for mediation of the influences of estrogen on neuronal differentiation, survival, and plasticity.

3.308 A novel monoclonal antibody recognizes lysosome-like structures and reflects regional and age-related differences in the rat dentate gyrus
Maeda, S. Et al
Neurosci. Lett., 330, 275-279 (2002)

The granule cells (GCs) of dentate gyrus exhibit regionally specific morphology, and continue to be born and to develop well into adult life. We used a novel monoclonal antibody, MAb2G7, elicited by immunization of a mouse with a microsome fraction of the hippocampus, to evaluate regional and age-related differences in GCs immunohistochemically. Weak cytoplasmic reactions were observed in many neurons, but intense MAb2G7-positive dots were observed only in GCs. Using electron microscopy, we observed that these dots were localized in the internal droplets of secondary lysosome-like structures in GCs. The MAb2G7-positive granules were quantitatively analyzed in young adult and middle-aged rats. Larger numbers of reactive granules were observed in the infrapyramidal blade (IPB) than in the suprapyramidal blade (SPB) and the numbers of positive granules were proportionally reduced in the two areas in middle-aged rats. The changes in the MAb2G7 immunoreactivity may reflect different activation or neurogeneration of GCs in the IPB versus the SPB, and in middle-aged versus young adult rats.

3.309 Nuclear translocation of extracellular superoxide dismutase
Ookawara, T. et al
Biochem. Biophys. Res. Comm., 296, 54-61 (2002)

Histochemical examination of mouse tissues showed nuclear staining of extracellular superoxide dismutase (EC-SOD), and the nuclear translocation of EC-SOD was also confirmed in cultured cells that had been transfected with its gene, as shown by immunohistochemistry and Western blot analysis. The EC-SOD which was secreted into the medium was incorporated into 3T3-L1 cells and a significant fraction of the material taken up was localized in the nucleus. Site-directed mutagenesis indicated that the heparin-binding domain of EC-SOD functions as the nuclear localization signal. These results suggest that the mechanism of the nuclear transport of EC-SOD involves a series of N-terminal signal peptide- and C-terminal heparin-binding domain-dependent processes of secretion, re-uptake and the subsequent nuclear translocation. The findings herein provide support for the view that the role of EC-SOD is to protect the genome DNA from damage by reactive oxygen species and/or the transcriptional regulation of redox-sensitive gene expression.

3.310 Initial steps of *Shigella* infection depend on the cholesterol/shingolipid raft-mediated CD44-IpaB interaction
Lafont, F. Et al
The EMBO J., 21(17), 4449-4457 (2002)

Shigellosis is an acute inflammatory bowel disease caused by the enteroinvasive bacterium *Shigella*. Upon host cell–*Shigella* interaction, major host cell signalling responses are activated. Deciphering the initial molecular events is crucial to understanding the infectious process. We identified a molecular complex involving proteins of both the host, CD44 the hyaluronan receptor, and *Shigella*, the invasin IpaB, which partitions during infection within specialized membrane microdomains enriched in cholesterol and sphingolipids, called rafts. We also document accumulation of cholesterol and raft-associated proteins at *Shigella* entry foci. Moreover, we report that *Shigella* entry is impaired after cholesterol depletion using methyl-ß-cyclodextrin. Finally, we find that *Shigella* is less invasive in sphingosid-based lipid-deficient cell lines, demonstrating the involvement of sphingolipids. Our results show that rafts are implicated in *Shigella* binding and entry, suggesting that raft-associated molecular machineries are engaged in mediating the cell signalling response required for the invasion process.

3.311 Plasma membrane phospholipid scamblase 1 is enriched in lipid rafts and interacts with the epidermal growth factor receptor
Sun, J., Nanjundan, M., Pike, L.J., Wiedmer, T. and Sims, P.J.
Biochemistry, 41, 6338-6345 (2002)

We have identified physical and functional interactions between the epidermal growth factor (EGF) receptor and phospholipid scramblase 1 (PLSCR1), an endofacial plasma membrane protein proposed to affect phospholipid organization. PLSCR1, a palmitoylated protein, was found to partition with the EGF receptor in membrane lipid rafts. Cell stimulation with EGF transiently elevated Tyr-phosphorylation of PLSCR1, peaking at 5 min. Although PLSCR1 is known substrate of c-Ab1 (Sun, J., et al. (2001) *J. Biol. Chem. 276, 28984-28990)*, the Ab1 inhibitor STI571 did not substantially affect its EGF-dependent phosphorylation, suggesting PLSCR1 is a substrate of the EGF receptor kinase, or another EGF-activated kinase. Coinciding with phosphorylation, there was a transient increase in physical association of PLSCR1 with both the EGF receptor and the adapter protein Shc, as determined by immunoprecipitation and Western blotting. Confocal immunofluorescence analysis revealed that EGF initiates rapid internalization of both the EGF receptor and PLSCR1, with trafficking into both distinct and common endosomal pools. These data also suggested that whereas the EGF receptor is ultimately degraded, much of the endocytosed PLSCR1 is recycled to the cell surface with 3 h after EGF treatment. Consistent with this interpretation. Western blotting revealed neither ubiquitination nor proteolysis of PLSCR1 under these conditions, whereas the ubiquitination and degradation of the EGF receptor were rapidly confirmed. Finally, stimulation with EGF was also found to markedly increase the total cellular expression of PLSCR1, suggesting that in addition to its initial interactions with activated EGF receptor, PLSCR1 may also contribute to posttranscriptional effector pathway(s) mediating the cellular response to EGF.

3.312 Truncated soluble Nogo receptor binds Nogo-66 and blocks inhibition of axon growth by myelin
Fournier, A.E., Gould, G.C., Liu, B.P. and Strittmatter, S.M.
*J. Neurosci., **22(20)**, 8876-8883 (2002)*

CNS myelin contains axon outgrowth inhibitors, such as Nogo, that restrict regenerative growth after injury. An understanding of the mechanism of Nogo signaling through its receptor (NgR) is critical to developing strategies for overcoming Nogo-mediated inhibition. Here we analyze the function of NgR domains in outgrowth inhibition. Analysis of alkaline phosphatase (AP)-Nogo binding in COS-7 cells reveals that the leucine-rich repeat domain is necessary and sufficient for Nogo binding and NgR multimerization. Viral infection of embryonic day 7 chick retinal ganglion cells with mutated NgR demonstrates that the NgR C-terminal domain is required for inhibitory signaling but not ligand binding. The NgR glycosylphosphatidylinositol domain is not essential for inhibitory signaling but may facilitate Nogo responses. From this analysis, we have developed a soluble, truncated version of the Nogo receptor that antagonizes outgrowth inhibition on both myelin and Nogo substrates. These data suggest that NgR mediates a significant fraction of myelin inhibition of axon outgrowth.

3.313 Cell surface polarization during yeast mating

Bagnat, M. and Simons, K.

Proc. Natl. Acad. Sci., 99(22), 14283-14188 (2002)

Exposure to mating pheromone in haploid *Saccharomyces cerevisiae* cells results in the arrest of the cell cycle, expression of mating-specific genes, and polarized growth toward the mating partner. Proteins involved in signaling, polarization, cell adhesion, and fusion are localized to the tip of the mating cell (shmoo) where fusion will eventually occur. The mechanisms ensuring the correct targeting and retention of these proteins are poorly understood. Here we show that in pheromone-treated cells, a reorganization of the plasma membrane involving lipid rafts results in the retention of proteins at the tip of the mating projection, segregated from the rest of the membrane. Sphingolipid and ergosterol biosynthetic mutants fail to polarize proteins to the tip of the shmoo and are deficient in mating. Our results show that membrane microdomain clustering at the mating projection is involved in the generation and maintenance of polarity during mating.

3.314 RNA incorporation is critical for retroviral particle integrity after cell membrane assembly of Gag complexes

Wang, S-W. and Aldovini, A.

J. Virol., 76(23), 11853-11865 (2002)

The nucleocapsid (NC) domain of retroviruses plays a critical role in specific viral RNA packaging and virus assembly. RNA is thought to facilitate viral particle assembly, but the results described here with NC mutants indicate that it also plays a critical role in particle integrity. We investigated the assembly and integrity of particles produced by the human immunodeficiency virus type 1 M1-2/BR mutant virus, in which 10 of the 13 positive residues of NC have been replaced with alanines and incorporation of viral genomic RNA is virtually abolished. We found that the mutations in the basic residues of NC did not disrupt Gag assembly at the cell membrane. The mutant Gag protein can assemble efficiently at the cell membrane, and viral proteins are detected outside the cell as efficiently as they are for the wild type. However, only ~10% of the Gag molecules present in the supernatant of this mutant sediment at the correct density for a retro-viral particle. The reduction of positive charge in the NC basic domain of the M1-2/BR virus adversely affects both the specific and nonspecific RNA binding properties of NC, and thus the assembled Gag poly-protein does not bind significant amounts of viral or cellular RNA. We found a direct correlation between the % of Gag associated with sedimented particles and the amount of incorporated RNA. We con-clude that RNA binding by Gag, whether the RNA is viral or not, is critical to retroviral particle integrity after cell membrane assembly and is less important for Gag-Gag interactions during particle assembly and release.

3.315 Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes

Nebl, T. et al

J. Biol. Chem., 277(45), 43999-43409 (2002)

Plasma membranes are organized into functional domains both by liquid-ordered packing into "lipid rafts," structures that resist Triton extraction, and by attachments to underlying cytoskeletal proteins in assemblies called "membrane skeletons." Although the actin cytoskeleton is implicated in many lipid raft-mediated signaling processes, little is known about the biochemical basis for actin involvement. We show here that a subset of plasma membrane skeleton proteins from bovine neutrophils co-isolates with cholesterol-rich, detergent-resistant membrane fragments (DRMs) that exhibit a relatively high buoyant density in sucrose (DRM-H; *d* ~1.16 g/ml). By using matrix-assisted laser desorption/ionization time of flight and tandem mass spectrometry, we identified 19 major DRM-H proteins. Membrane skeleton proteins include fodrin (nonerythroid spectrin), myosin-IIA, myosin-IG, α-actinin 1, α-actinin 4, vimentin, and the F-actin-binding protein, supervillin. Other DRM-H components include lipid raft-associated integral membrane proteins (stomatin, flotillin 1, and flotillin 2), extracellular surface-bound and glycophosphatidylinositol-anchored proteins (IgM, membrane-type 6 matrix metalloproteinase), and intracellular dually acylated signaling proteins (Lyn kinase, $G\alpha_{i-2}$). Consistent with cytoskeletal association, most DRM-H-associated flotillin 2, Lyn, and $G\alpha_{i-2}$ also resist extraction with 0.1 M octyl glucoside. Supervillin, myosin-IG, and myosin-IIA resist extraction with 0.1 M sodium carbonate, a treatment that removes all detectable actin, suggesting that these cytoskeletal proteins are proximal to the DRM-H bilayer. Binding of supervillin to the DRM-H fragments is confirmed by co-immunoaffinity purification. In spreading neutrophils, supervillin localizes with F-actin in cell extensions and in discrete basal puncta that partially overlap with $G\alpha_i$ staining. We

suggest that the DRM-H fraction represents a membrane skeleton-associated subset of leukocyte signaling domains.

3.316 Presence of detergent-resistant microdomains in lysosomal membranes
Taute, A. et al
Biochem. Biophys. Res. Comm., ***298(3)***, *5-9 (2002)*

We examined the association of acetyl-CoA:α -glucosaminide *N*-acetyltransferase, a lysosomal enzyme participating in the degradation of heparan sulfate with other components of the lysosomal membrane. We prepared lysosomal membranes from human placenta and treated them with zwitterionic and non-ionic detergents. Membrane proteins were solubilized either in the presence of CHAPS at room temperature or of Triton X-100 at 4 °C. The CHAPS-containing extract was subjected to gel filtration in a column with the nominal size exclusion of 0.6 MDa. Under these conditions the enzyme fractionated near the void volume. To examine the association of the enzyme with detergent-resistant lipid microdomains, the extract that had been prepared with Triton X-100 was subjected to flotation in a density gradient medium. After centrifug-ation, a major portion of the activity of the acetyltransferase was found at the top of the gradient along with the bulk of alkaline phosphatase. Alkaline phosphatase is a glycosylphosphatidylinositol-anchored protein; possibly a contaminant in the lysosomal fraction originating from the plasma membrane and adventitiously an internal control for the flotation in the gradient. In contrast, acetyltransferase is a genuine lysosomal protein that obligatorily spans the membrane since it transfers acetyl residues from acetyl-CoA in cytosol to glucosaminyl residues in heparan sulfate fragments in the lysosomal matrix. To our knowledge this is the first report on association of a lysosomal membrane protein with detergent-resistant membrane microdomains or rafts.

3.317 A specific binding protein/receptor for 1α,25-dihydroxyvitamin D_3 is present in an intestinal caveolae membrane fraction
Norman, A.W., Olivera, C.J., Silva, F.R.M.B. and Bishop, J.E.
Biochem. Biophys. Res. Comm., ***298(3)***, *414-419 (2002)*

The steroid hormone 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] produces biological responses by interaction with both a well-characterized nuclear receptor (VDR_{nuc}) to regulate gene transcription and with an as-yet uncharacterized membrane-associated protein/receptor (VDR_{mem}) to generate a variety of rapid, non-genotropic responses. We report for the first time that [^{3}H]1α,25$(OH)_2D_3$ binds with high affinity to a chick duodenal caveolae-enriched membrane fraction (CMF) isolated without the use of detergents. Caveolae are plasma membrane invaginations implicated in signal transduction and molecular transport processes. Using the CMF fraction as a possible source of VDR_{mem}, we found that the in vitro binding of [^{3}H]1α,25$(OH)_2D_3$ was ligand dependent and saturable; the K_D and B_{max} were 1.3±0.6 nM and 29±11 fmol 1,25$(OH)_2D_3$/mg protein (n=17), respectively. Immunoblot analysis of the CMF confirms the presence of caveolin-1, a marker protein for membranes with caveolae. Therefore, chick CMF may represent a good source for isolation and characterization of the putative VDR_{mem} for 1α,25$(OH)_2D_3$.

3.318 Novel non-labile covalent binding of sulfamethoxazole reactive metabolites to cultured human lymphoid cells

Summan, M. and Cribb, A.E.

Chemico-Biol. Interactions, 142, 155-173 (2002)

Sulfamethoxazole (SMX) causes rare hypersensitivity syndrome reactions characterized by fever and multi-organ toxicity. Covalent binding of SMX reactive metabolites to cellular proteins has been demonstrated but the link between cytotoxicity and targets of covalent binding has not been explored. We therefore invest-igated the relationship between covalent binding of the reactive SMX-hydroxylamine (SMX-HA) metabo-lite, and its cytotoxicity to a hystiocytic lymphoma (U937) cell line. Incubation of U937 cells with 0–1 mM SMX-HA for 3 h resulted in dose-dependent cytotoxicity, as assessed by tetrazolium dye conversion at 24 h. SMX-HA caused dose-dependent covalent binding to cellular proteins as assessed by immunoblotting with SMX antisera at 3 and 24 h. Covalent binding was predominantly to proteins of approximately 45, 59 and 75 kDa, but other targets were also observed. The relative extent of binding to proteins was significantly different from the relative cytotoxicity at 24 h. Further, cells surviving at 24 h also had extensive covalent binding. Covalent binding was observed under reducing (β-mercaptoethanol) and non-reducing conditions to plasma membrane and microsomal but not cytosolic proteins. This non-labile covalent binding has not been previously reported. These observations suggest that extensive covalent binding does not necessarily lead to cell death, allowing the accumulation of potentially immunogenic drug-protein conjugates. These observations in whole cells may be relevant to the immunopathogenesis of SMX hypersensitivity syndrome reactions.

3.319 Molecular mechanism for orienting membrane and actin dynamics to nascent cell-cell contacts in epithelial cells

Hansen, M.D., Ehrlich, J.S. and Nelson, W.J.

J. Biol. Chem., 277(47), 45371-45376 (2002)

The small GTPase Rac1 has been implicated in regulation of cell migration and cell-cell adhesion in epithelial cells. Little is known, however, about the spatial and temporal coordination of Rac1 activity required to balance these competing processes. We fractionated endogenous Rac1-containing protein complexes from membranes of Madin-Darby canine kidney cells and identified three major complexes comprising a Rac1·PAK (p21-activated kinase) complex, and 11 S and 16 S Rac1 complexes. Significantly, Rac1 shifts from the 11 S to a 16 S particle during initiation of cell-cell adhesion. This shift may reflect a diffusion trapping mechanism by which these Rac1 complexes are localized to cadherin-mediated cell-cell contacts through an interaction with annexin II.

3.320 Regulated expression and intracellular localization of cystatin F in human U937 cells
Nathanson, C-M, Wasselius, J., Wallin, H. and Abrahamson, M.
*Eur. J. Biochem., **269**, 5502-5511 (2002)*

Cystatin F is a cysteine peptidase inhibitor recently discovered in haematopoietic cells by cDNA cloning. To further investigate the expression, distribution and properties of the native human inhibitor the promyeloid cell line U937 has been studied. The cells expressed relatively large quantities of cystatin F, which was found both secreted and intracellularly. The intracellular levels were unusually high for a secreted cystatin (≈25% of the cystatin F in 2- or 4-day culture medium). By contrast, U937 cells contained only 3–4% of the related inhibitor, cystatin C. Cystatin F purified from lysates of U937 cells showed three major forms carrying two, one or no carbohydrate chains. Immunocytochemistry demonstrated a marked cytoplasmic cystatin F staining in a granular pattern. Double staining with a marker for endoplasmic reticulum revealed no colocal-ization for cystatin F. Analysis of the promoter region of the cystatin F gene (*CST7*) showed that it, like that of the cystatin C gene (*CST3*), is devoid of typical TATA- and CAAT-box elements. In contrast to the cyst-atin C promoter, it does not contain multiple Sp1 binding sites, but has a unique site for C/EBPα, possibly explaining the restricted expression of the cystatin F gene. Cells stimulated with all-*trans* retinoic acid to differentiate them towards a granulocytic pathway, showed a strong (≈18-fold) down-regulation of intra-cellular cystatin F and almost abolished secreted levels of the inhibitor. Stimulation with tetradecanoyl phorbol acetate, causing monocytic differentiation, also resulted in down-regulation (two fold to threefold) of cystatin F expression, whereas the cystatin C expression was essentially unaltered in both experiments. The results suggest that cystatin F as an intracellular cysteine peptidase inhibitor with readily regulated expression, may be a candidate to control the cysteine peptidase activity known to be essential for antigen presentation in different blood cell lineages.

3.321 The Ccz1-Mon1 protein complex is required for the late step of multiple vacuole delivery pathways
Wang, C-W, Stromhaug, P.E., Shima, J. and Klionsky, J.
*J. Biol. Chem., **277(49)**, 47917-47927 (2002)*

Mon1 and Ccz1 were identified from a gene deletion library as mutants defective in the vacuolar import of aminopeptidase I (Ape1) via the cytoplasm to vacuole targeting (Cvt) pathway. The *mon1Δ* and *ccz1Δ* strains also displayed defects in autophagy and pexophagy, degradative pathways that share protein machinery and mechanistic features with the biosynthetic Cvt pathway. Further analyses indicated that Mon1, like Ccz1, was required in nearly all membrane-trafficking pathways where the vacuole represented the terminal acceptor compartment. Accordingly, both deletion strains had kinetic defects in the biosynthetic delivery of resident vacuolar hydrolases through the CPY, ALP, and MVB pathways. Biochemical and microscopy studies suggested that Mon1 and Ccz1 functioned after transport vesicle formation but before (or at) the fusion step with the vacuole. Thus, *ccz1Δ* and *mon1Δ* are the first mutants identified in screens for the Cvt and Apg pathways that accumulate precursor Ape1 within completed cytosolic vesicles. Subcellular fractionation and co-immunoprecipitation experiments confirm that Mon1 and Ccz1 physically interact as a stable protein complex termed the Ccz1-Mon1 complex. Microscopy of Ccz1 and Mon1 tagged with a fluorescent marker indicated that the Ccz1-Mon1 complex peripherally associated with a perivacuolar compartment and may attach to the vacuole membrane in agreement with their proposed function in fusion.

3.322 Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis

Pike, L.J., Han, X., Chung, K-N and Gross, R.W.
Biochemistry, ***41***, *2075-2088 (2002)*

Lipid rafts are specialized cholesterol-enriched membrane domains that participate in cellular signaling processes. Caveolae are related domains that become invaginated due to the presence of the structural protein, caveolin-1. In this paper, we use electrospray ionization mass spectrometry (ESI/MS) to quantitatively compare the phospholipids present in plasma membranes and nondetergent lipid rafts from caveolin-1-expressing and nonexpressing cells. Lipid rafts are enriched in cholesterol and sphingomyelin as compared to the plasma membrane fraction. Expression of caveolin-1 increases the amount of cholesterol recovered in the lipid raft fraction but does not affect the relative proportions of the various phospholipid classes. Surprisingly, ESI/MS demonstrated that lipid rafts are enriched in plasmenylethanolamines, particularly those containing arachidonic acid. While the total content of anionic phospholipids was similar in plasma membranes and nondetergent lipid rafts, the latter were highly enriched in phosphatidylserine but relatively depleted in phosphatidylinositol. Detergent-resistant membranes made from the same cells showed a higher cholesterol content than nondetergent lipid rafts but were depleted in anionic phospholipids. In addition, these detergent-resistant membranes were not enriched in arachidonic acid-containing ethanolamine plasmalogens. These data provide insight into the structure of lipid rafts and identify potential new roles for these domains in signal transduction.

3.323 Endoproteolysis of presenilin in vitro: inhibition by γ-secretase inhibitors

Campbell, W.A., Iskandar, M-K., Reed, M.L.O. and Xia, W.
Biochemistry, ***41***, *3372-3379 (2002)*

The final proteolytic step to generate the amyloid beta-protein (Abeta) of Alzheimer's disease (AD) from beta-amyloid precursor protein (APP) is achieved by presenilin (PS)-dependent gamma-secretase cleavage. AD-causing mutations in PS1 and PS2 result in a selective and significant increase in production of the more amyloidogenic Abeta42 peptide. PS1 and PS2 undergo endoproteolysis by an unknown enzyme termed presenilinase to generate the functional complex of N- and C-terminal fragments (NTF/CTF). To investigate the endoproteolytic activity that generates active PS, we used a mammalian cell-free system that allows de novo human PS NTF and CTF generation. PS NTF and CTF generation in vitro was observed in endoplasmic reticulum (ER)-enriched fractions of membrane vesicles and to a lesser extent in Golgi/trans-Golgi-network (TGN)-enriched fractions. AD-causing mutations in PS1 and PS2 did not alter de novo generation of PS fragments. Removal of peripheral membrane-associated and cytosolic proteins did not prevent de novo generation of fragments, indicating that presenilinase activity corresponds to an integral membrane protein. Among several general inhibitors of different protease classes that blocked the presenilinase activity, pepstatin A was the most potent inhibitor. Screening available transition state analogue gamma-secretase inhibitors led to the identification of two compounds that were able to prevent the de novo generation of PS fragments, with an expected inhibition of Abeta generation. Our studies provide a biochemical approach to characterize and identify this elusive presenilinase.

3.324 Annular α-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes
Ding, T.T., Lee, S-J., Rochet, J-C. and Lansbury, Jr., P.T.
*Biochemistry, **41**, 10209-10217 (2002)*

The Parkinson's disease substantia nigra is characterized by the loss of dopaminergic neurons and the presence of cytoplasmic fibrillar Lewy bodies in surviving neurons. The major fibrillar protein of Lewy bodies is alpha-synuclein. Two point mutations in the alpha-synuclein gene are associated with autosomal-dominant Parkinson's disease (FPD). Studies of the in vitro fibrillization behavior of the mutant proteins suggest that fibril precursors, or alpha-synuclein protofibrils, rather than the fibrils, may be pathogenic. Atomic force microscopy (AFM) revealed two distinct forms of protofibrillar alpha-synuclein: rapidly formed spherical protofibrils and annular protofibrils, which were produced on prolonged incubation of spheres. The spherical protofibrils bound to brain-derived membrane fractions much more tightly than did monomeric or fibrillar alpha-synuclein, and membrane-associated annular protofibrils were observed. The structural features of alpha-synuclein annular protofibrils are reminiscent of bacterial pore-forming toxins and are consistent with their porelike activity in vitro. Thus, abnormal membrane permeabilization may be a pathogenic mechanism in PD.

3.325 Characterization of isolated acidocalcisomes from *Toxoplasma gondii* Tachyzoites reveals a novel pool of hydrolysable polyphosphate
Rodrigues, C.O., Ruiz, F.A., Rohloff, P., Dcott, D.A. and Moreno, S.N.J.
J. Biol. Chem., 277(50), 48650-48656 (2002)

Toxoplasma gondii tachyzoites were fractionated by modification of an **iodixanol** density gradient method previously used for acidocalcisome isolation from *Trypanosoma cruzi* epimastigotes. Fractions were characterized using electron microscopy, x-ray microanalysis, and enzymatic markers, and it was demonstrated that the heaviest (pellet) fraction contains electron-dense vacuoles rich in phosphorus, calcium, and magnesium, as found before for acidocalcisomes. Staining with 4',6-diamidino-2-phenylindole (DAPI) indicated that poly- phosphate (polyP) was preferentially localized in this fraction together with pyrophosphate (PP_i). Using an enzyme-based method, millimolar levels (in terms of P_i residues) of polyP chains of less than 50 residues long and micromolar levels in polyP chains of about 700-800 residues long were found to be preferentially localized in this fraction. The fraction also contained the pyrophosphatase and polyphosphatase activities characteristic of acidocalcisomes. Western blot analysis using antibodies against proteins from micronemes, dense granules, rhoptries, and plasma membrane showed that the acidocalcisomal fraction was not contaminated by these other organelles. *T. gondii* polyP levels rapidly decreased upon exposure of the parasites to a calcium ionophore (ionomycin), to an inhibitor of the V-H^+-ATPase (bafilomycin A_1), or to the alkalinizing agent NH_4Cl. These changes were in parallel to an increase in intracellular Ca^{2+} concentration, suggesting a close association between polyP hydrolysis and Ca^{2+} release from the acidocalcisome. These results provide a useful method for the isolation and characterization of acidocalcisomes, showing that they are distinct from other previously recognized organelles present in *T. gondii*, and provide evidence for the role of polyP metabolism in response to cellular stress.

3.326 Formation of mutually exclusive Rab11 complexes with members of the family of Rab11-interacting proteins regulates Rab11 endocytic targeting and function
Meyers, J.M. and Prekeris, R.
J. Biol. Chem., 277(50), 49003-49010 (2002)

Several Rabs, including Rab11, regulate the traffic and sorting of proteins in the endosomal pathway. Recently, six novel Rab11 family interacting proteins (FIPs) were identified. Although they share little overall sequence homology, all FIPs contain a conserved Rab11-binding domain. Here we investigate the role of FIPs as Rab11-targeting proteins and show that the Rab11-binding domain assumes an α-helical structure, with the conserved residues forming a hydrophobic Rab11-binding patch. This hydrophobic patch mediates the formation of mutually exclusive complexes between Rab11 and various members of FIP protein family. Furthermore, the formation of Rab11/FIP complexes regulates Rab11 localization by recruiting it to distinct endocytic compartments. Thus, we propose that Rab11/FIP complexes serve as targeting patches, regulating Rab11 localization and recruitment of additional cellular factors to different endocytic compartments.

3.327 A specific structural requirement for ergosterol in long-chain fatty acid synthesis mutants important for maintaining raft domains in yeast

Eisenkolb, M., Zenzmaier, C., Leitner, E., and Schneiter, R.
*Mol. Biol. Cell, **13**, 4414-4428 (2002)*

Fungal sphingolipids contain ceramide with a very-long-chain fatty acid (C26). To investigate the physiological significance of the C26-substitution on this lipid, we performed a screen for mutants that are synthetically lethal with *ELO3*. Elo3p is a component of the ER-associated fatty acid elongase and is required for the final elongation cycle to produce C26 from C22/C24 fatty acids. *elo3Δ* mutant cells thus contain C22/C24- instead of the natural C26-substituted ceramide. We now report that under these conditions, an otherwise nonessential, but also fungal-specific, structural modification of the major sterol of yeast, ergosterol, becomes essential, because mutations in *ELO3* are synthetically lethal with mutations in *ERG6*. Erg6p catalyzes the methylation of carbon atom 24 in the aliphatic side chain of sterol. The lethality of an *elo3Δ erg6Δ* double mutant is rescued by supplementation with ergosterol but not with cholesterol, indicating a vital structural requirement for the ergosterol-specific methyl group. To characterize this structural requirement in more detail, we generated a strain that is temperature sensitive for the function of Erg6p in an *elo3Δ* mutant background. Examination of raft association of the GPI-anchored Gas1p and plasma membrane ATPase, Pma1p, in the conditional *elo3Δ erg6^{ts}* double mutant, revealed a specific defect of the mutant to maintain raft association of preexisting Pma1p. Interestingly, in an *elo3Δ* mutant at 37°C, newly synthesized Pma1p failed to enter raft domains early in the biosynthetic pathway, and upon arrival at the plasma membrane was rerouted to the vacuole for degradation. These observations indicate that the C26 fatty acid substitution on lipids is important for establishing raft association of Pma1p and stabilizing the protein at the cell surface. Analysis of raft lipids in the conditional mutant strain revealed a selective enrichment of ergosterol in detergent-resistant membrane domains, indicating that specific structural determinants on both sterols and sphingolipids are required for their association into raft domains.

3.328 Lipid-dependent subcellular relocalization of the acyl chain desaturase in yeast

Tatzer, V., Zellnig, G., Kohlwein, S.D. and Schneiter, R.
Mol. Biol. Cell, 13, 4429-4442 (2002)

The degree of acyl chain desaturation of membrane lipids is a critical determinant of membrane fluidity. Temperature-sensitive mutants of the single essential acyl chain desaturase, Ole1p, of yeast have previously been isolated in screens for mitochondrial inheritance mutants (Stewart, L.C., and Yaffe, M.P. (1991). J. Cell Biol. *115*, 1249-1257). We now report that the mutant desaturase relocalizes from its uniform ER distribution to a more punctuate localization at the cell periphery upon inactivation of the enzyme. This relocalization takes place within minutes at nonpermissive conditions, a time scale at which mitochondrial morphology and inheritance is not yet affected. Relocalization of the desaturase is fully reversible and does not affect the steady state localization of other ER resident proteins or the kinetic and fidelity of the secretory pathway, indicating a high degree of selectivity for the desaturase. Relocalization of the desaturase is energy independent but is lipid dependent because it is rescued by supplementation with unsaturated fatty acids. Relocalization of the desaturase is also observed in cells treated with inhibitors of the enzyme, indicating that it is independent of temperature-induced alterations of the enzyme. In the absence of desaturase function, lipid synthesis continues, resulting in the generation of lipids with saturated acyl chains. A model is discussed in which the accumulation of saturated lipids in a microdomain around the desaturase could induce the observed segregation and relocalization of the enzyme.

3.329 The mechanism of γ-secretase activities through high molecular weight complex formation pof presenilins is conserved in *Drosophila melanogaster* and mammals
Takasugi, N., Takahashi, Y., Morohashi, Y. and Tomita, T.
J. Biol. Chem., 277(51), 50198-50205 (2002)

Mutations in presenilin 1 (*PS1*) and *PS2* genes contribute to the pathogenesis of early onset familial Alzheimer's disease by increasing secretion of the pathologically relevant Aβ42 polypeptides. PS genes are also implicated in Notch signaling through proteolytic processing of the Notch receptor in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals. Here we show that *Drosophila* PS (Psn) protein undergoes endoproteolytic cleavage and forms a stable high molecular weight (HMW) complex in *Drosophila* S2 or mouse neuro2a (N2a) cells in a similar manner to mammalian PS. The loss-of-function recessive point mutations located in the C-terminal region of Psn, that cause an early pupal-lethal phenotype resembling *Notch* mutant *in vivo*, disrupted the HMW complex formation, and abolished γ-secretase activities in cultured cells. The overexpression of Psn in mouse embryonic fibroblasts lacking *PS1* and *PS2* genes rescued the Notch processing. Moreover, disruption of the expression of Psn by double-stranded RNA-mediated interference completely abolished the γ-secretase activity in S2 cells. Surprisingly, γ-secretase activity dependent on wild-type Psn was associated with a drastic overproduction of Aβ1-42 from human βAPP in N2a cells, but not in S2 cells. Our data suggest that the mechanism of γ-secretase activities through formation of HMW PS complex, as well as its abolition by loss-of-function mutations located in the C terminus, are highly conserved features in *Drosophila* and mammals.

3.330 Axonal transport of mitochondria to synapses depends on Milton, a novel *Drosophilia* protein
Stowers, R.S., Megeath, L.J., Gorska-Andrzejak, J., Meinertzhagen, I.A. and Schwartz, T.L.
Neuron, 36, 1063-1077 (2002)

A protein required to localize mitochondria to *Drosophila* nerve terminals has been identified genetically. Photoreceptors mutant for *milton* show aberrant synaptic transmission despite normal phototransduction. Without Milton, synaptic terminals and axons lack mitochondria, although mitochondria are numerous in neuronal cell bodies. In contrast, synaptic vesicles continue to be transported to and concentrated at synapses. Milton protein is associated with mitochondria and is present primarily in axons and synapses. A likely explanation of the apparent trafficking defect is offered by the coimmunoprecipitation of Milton and kinesin heavy chain. Transfected into HEK293T cells, Milton induces a redistribution of mitochondria within the cell. We propose that Milton is a mitochondria-associated protein required for kinesin-mediated transport of mitochondria to nerve terminals.

3.331 The organizing principle in the formation of the T cell receptor-CD3 complex
Call, M.E., Pyrdol, J., Wiedmann, M. and Wucherpfennig, K.W.
*Cell, **111**, 967-979 (2002)*

The T cell receptor (TCR) serves a critical function in the immune system and represents one of the most complex receptor structures. A striking feature is the presence of nine highly conserved, potentially charged residues in the transmembrane helices. Previous models have attempted to explain assembly based on pairwise interactions of these residues. Using a novel method for the isolation of intact radiolabeled protein complexes, we demonstrate that one basic and two acidic transmembrane residues are required for the assembly of each of the three signaling dimers with the TCR. This remarkable three-helix arrangement applies to all three assembly steps and represents the organizing principle for the formation of this intricate receptor structure.

3.332 Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDA and phospholipase Cγ1 are required for NF-κB activation and lipid raft recruitment of protein kinase Cθ induced by T cell costimulation

Dienz, O. et al

J. Immunol., ***169****, 365-372 (2002)*

The NF-κB activation pathway induced by T cell costimulation uses various molecules including Vav1 and protein kinase C (PKC)*θ*. Because Vav1 inducibly associates with further proteins including phospholipase C (PLC)γ 1 and Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76), we investigated their role for NF-κB activation in Jurkat leukemia T cell lines deficient for expression of these two proteins. Cells lacking SLP-76 or PLCγ1 failed to activate NF-κB in response to T cell costimulation. In contrast, replenishment of SLP-76 or PLCγ1 expression restored CD3/CD28-induced IκB kinase (IKK) activity as well as NF-κB DNA binding and transactivation. PKC*θ* activated NF-κB in SLP-76- and PLCγ1-deficient cells, showing that PKC*θ* is acting further downstream. In contrast, Vav1-induced NF-κB activation was normal in SLP-76$^-$ cells, but absent in PLCγ1$^-$ cells. CD3/CD28-stimulated recruitment of PKCθ and IKKγ to lipid rafts was lost in SLP-76- or PLCγ1-negative cells, while translocation of Vav1 remained unaffected. Accordingly, recruitment of PKC*θ* to the immunological synapse strictly relied on the presence of SLP-76 and PLCγ1, but synapse translocation of Vav1 identified in this study was independent from both proteins. These results show the importance of SLP-76 and PLCγ1 for NF-κB activation and raft translocation of PKC*θ* and IKKγ.

3.333 Genetic analysis of iron citrate toxicity in yeast: implications for mammalian iron homeostasis

Chen, O.S., Hemenway, S. and Kaplan, J.

Proc. Natl. Acad. Sci., USA, ***99(26)****, 16922-16927 (2002)*

Deletion of the yeast homologue of frataxin, *YFH1*, results in mitochondrial iron accumulation and respiratory deficiency (petite formation). We used a genetic screen to identify mutants that modify iron-associated defects in respiratory activity in Δ*yfh1* cells. A deletion in the peroxisomal citrate synthase *CIT2* in Δ*yfh1* cells decreased the rate of petite formation. Conversely, overexpression of *CIT2* in Δ*yfh1* cells increased the rate of respiratory loss. Citrate toxicity in Δ*yfh1* cells was dependent on iron but was independent of mitochondrial respiration. Citrate toxicity was not restricted to iron-laden mitochondria but also occurred when iron accumulated in cytosol because of impaired vacuolar iron storage. These results suggest that high levels of citrate may promote iron-mediated tissue damage.

3.334 Regulated secretion and subcellular distribution of rabbit lacrimal gland alpha-fucosidases
Gierow, J., Andersson, S.V. and Sjögren, E.C.
Invest. Ophthamol. Vis. Sci., ***43***, *E-Abstract 3125 (2002)*

Purpose: Our previous results have indicated the presence of two forms of alpha-fucosidase, one acidic and one neutral, that are expressed and secreted by rabbit lacrimal acinar cells in primary culture (IOVS 42: s259, 2001). The purpose of the present study was to further characterize the two forms of alpha-fucosidase regarding their subcellular distribution and regulated secretion
Methods: Single acinar cells were isolated from female NZW rabbit lacrimal glands and cultured for 40 h on Matrigel, thus allowing the cells to re-organize into acinar-like structures. The cells were then rinsed and incubated at 37C for 1 h in the absence or presence of 0.1 mM carbachol. Media were collected and analyzed for alpha-fucosidase catalytic activity, at pH 4.0 and pH 7.0, using a 4-methyl umbelliferyl conjugate as substrate.Tissue and cultured cells were homogenized and fractionated by centrifugation yielding a soluble, a nuclear and a microsomal fraction. The membrane fractions were further fractionated by density gradient centrifugation on 10-30% Iodioxanol (**Optiprep**) gradients, and the resulting fractions were analyzed for fucosidase activity.
Results: Secretion of the acidic and the neutral form were both stimulated 5-fold by carbachol. Subcellular fractionation of tissue resulted in 60% recovery of both the acidic and the neutral fucosidase activity in the soluble fraction and 15% in the nuclear (low-speed) pellet, whereas fractionation of cultured cells resulted in a reversed distribution, the difference probably a result of the harsher homogenization procedures used for tissue. Density gradient centrifugation of the membrane fractions revealed the presence of at least three fucosidase-containing membrane populations: One fraction of low density of unknown origin, one high-density, potentially lysosomal population and one intermediate fraction, possibly Golgi-related.
Conclusion: No sigificant differences were observed between the distribution and secretion of the acidic and neutral forms of alpha-fucosidase, indicating that they are sequestered in the same endomembrane compartments and that they are under the same secretory control.

3.335 Human B1 and B2 bradykinin receptors and their agonists target caveolae-related lipid rafts to different degrees in HEK293 cells
Lamb, M.E., Zhang, C., Shea, T., Kyle, D.J. and Leeb-Lundberg, L.M.F.
Biochemistry, ***41***, *14340-14347 (2002)*

To address the targeting of G protein-coupled receptors to caveolae-related lipid rafts (CLR), we studied the human B2 (B2R) and B1 (B1R) bradykinin receptor subtypes in HEK293 cells. CLR were enriched on the basis of their unique buoyant density and composition of cholesterol, caveolin-1, and flotillin-1 but not clathrin. CLR contained B2R and B1R as determined by both receptor immunoblotting and the increase in specific activity of receptor agonist binding to cells at both 4 and 37 degrees C when binding was followed by CLR enrichment. B2R was highly enriched in this fraction, whereas B1R was not enriched. Furthermore, acid washing of cells prior to cell disruption minimally affected the CLR-associated B2R agonist binding, whereas it dissociated a major portion of the CLR-associated B1R agonist binding. In addition, when agonist binding at 4 degrees C was followed by an increase in the temperature to 37 degrees C, B2R agonist binding in CLR transiently increased, and this increase was dependent on the C-terminal domain. On the other hand, B1R agonist binding remained unchanged and was independent of the C-terminal domain. Our results show that B2R is constitutively targeted to CLR in HEK293 cells and appears to shuttle the agonist through these domains, whereas B1R may be there by default.

3.336 Hormone-induced subcellular redistribution of trimeric G proteins
Svobada, P. and Novotny, J.
*Cell. Mol. Life Sci., **59**, 501-512 (2002)*

Trimeric guanine nucleotide-binding proteins (G proteins) function as the key regulatory elements in a number of transmembrane signaling cascades where they convey information from agonist-activated receptors to effector molecules. The subcellular localization of G proteins is directly related to their functional role, i.e., the dominant portion of the cellular pool of G proteins resides in the plasma membrane. An intimate association of G protein subunits with the plasma membrane has been well known for a long time. However, results of a number of independent studies published in the past decade have indicated clearly that exposure of intact target cells to agonists results in subcellular redistribution of the cognate G proteins from plasma membranes to the light-vesicular membrane fractions, in internalization from the cell surface into the cell interior and in transfer from the membrane to the soluble cell fraction (high-speed supernatant), i.e. solubilization. Solubilization of G protein αsubunits as a consequence of stimulation of G protein-coupled receptors (GPCRs) with agonists has also been observed in isolated membrane preparations. The membrane -cytosol shift of G proteins was detected even after direct activation of these proteins by non-hydrolyzable analogues of GTP or by cholera toxin-induced ADP-ribosylation. In addition, prolonged stimulation of GPCRs with agonists has been shown to lead to down-regulation of the relevant G proteins. Together, these data suggest that G proteins might potentially participate in a highly complex set of events, which are gene-rally termed desensitization of the hormone response. Internalization, subcellular redistribution, solubiliza-tion, and down-regulation of trimeric G proteins may thus provide an additional means (i.e., beside receptor -based mechanisms) to dampen the hormone or neurotransmitter response after sustained (long-term) exposure.

3.337 Reversible depression of transcription during hibernation
van Breukelen, F. and Martin, S.L.
J. Comp. Physiol. B., 172, 355-361 (2002)

Mammalian hibernators downregulate processes of energy production and consumption while maintaining cellular homeostasis. Energetic costs of transcription must be balanced with demands for gene products. Data from nuclear run-on assays indicate transcriptional initiation is reduced two fold in torpid golden-mantled ground squirrels (*Spermophilus lateralis*) as compared to euthermic animals between bouts of torpor. In addition, elongation rates across the temperature range experienced by hibernators indicate a virtual arrest of transcription at the low body temperatures of torpor. Finally, there is no seasonal compensation or species-specific adaptation for increased elongational capacity in the cold. Thus, it appears that hibernators are not specifically adapted to continue transcription during torpor. Taken together, these data indicate that transcription arrests during torpor because of a moderate depression of initiation and a more severe inhibition of elongation, largely due to temperature effects. Restoration of euthermic body temperatures during the interbout arousals reverses this transcriptional depression and permits gene expression.

3.338 Myocilin is associated with mitochondria in human trabecular meshwork cells
Wentz-Hunter, K., Ueda, J., Shimuzi, N. and Yue, B.Y.J.T.
*J. Cell. Physiol., **190**, 46-53 (2002)*

The trabecular meshwork (TM) is a specialized tissue located at the chamber angle of the eye next to the cornea. This tissue is believed to be responsible for regulation of the aqueous humor outflow and control of the intraocular pressure (IOP). Alterations in functions of the TM may lead to IOP elevation and development of glaucoma, a major cause of blindness. The myocilin gene has recently been directly linked to open-angle glaucomas. The gene product was originally identified as a protein inducible in TM cells by treatment with glucocorticoids such as dexamethasone (DEX) and termed TIGR (TM inducible-glucocorticoid resp-onse). The exact nature and function of the myocilin protein so far still remain elusive. In this study, myocil-in was localized to the perinuclear region of both DEX-treated and control TM cells. Its distribution over-lapped considerably with that of mitochondria. Subcellular fractionation and Western blot analyses suggest-ed a rather extensive association of myocilin with mitochondria. The DEX-treated TM cells were found to undergo apoptosis, when exposed to anti-Fas antibody, to a signilicantly higher degree than the untreated control cells. It appears that the TM cell integrity remains intact after DEX treatment. However, the induc-ed myocilin or myocilin-mitochondria association seems to render the cells more susceptible to a second stress or challenge. This vulnerability may be the basis that ultimately leads to pathological consequences.

3.339 RVS161p and sphingolipids are required for actin repolarization following salt stress
Balguerie, A., Bagnat, M., Bonneu, M., Aigle, M. and Breton, A.M.
*Eukaryotic Cell, **1**, 1021-1031 (2002)*
In *Saccharomyces cerevisiae,* the actin cytoskeleton is depolarized by NaCl stress. In this study, the response was maximal after 30 mm, and then actin patches repolarized. Rvsl6lp was required for actin repolarization because the *rvs161Δ* mutant did not repolarize actin patches after growth in a salt medium. Mutations suppressing the rvsl6Th-related salt sensitivity all occurred in genes required for sphingolipid biosynthesis: *FEN1, SUR4, SUR2, SUR!,* and *IPT1*. These suppressors also suppressed *act1-l*-related salt sensitivity and the defect in actin repolarization of the *rvs161Δ* mutant, providing a link between sphingolipids and actin polarization. Indeed, deletion of the suppressor genes suppressed the *rvs161Δ* defect in actin repolarization in two ways: either actin was not depolarized at the wild-type level in a set of suppressor mutants, or actin was repolarized in the absence of Rvs16lp in the other suppressor mutants. Rvsl6lp was localized as cortical patches that concentrated at polarization sites, i.e., bud emergence and septa, and was found to be associated with lipid rafts. An important link between sphingolipids and actin polarization is that Rvsl61p was required for actin repolarization and was found to be located in lipid rafts.

3.340 Huntingtin-associated protein 1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate and functions in endosomal trafficking
Li, Y., Chin, L-S., Levey, A.L. and Li, L.
*J. Biol. Chem., **277(31)**, 28212-28221 (2002)*

Huntingtin-associated protein 1 (HAP1) is a novel protein of unknown function with a higher binding affinity for the mutant form of Huntington's disease protein huntingtin. Here we report that HAP1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a mammalian homologue of yeast vacuolar protein sorting protein Vps27p involved in the endosome-to-lysosome trafficking. This novel interaction was identified in a yeast two-hybrid screen using full-length Hrs as bait, and confirmed by *in vitro* binding assays and co-immunoprecipitation experiments. Deletion analysis reveals that the association of HAP1 with Hrs is mediated via a coiled-coil interaction between the central coiled-coil domains of both proteins. Immunofluorescence and subcellular fractionation studies show that HAP1 co-localizes with Hrs on early endosomes. Like Hrs, overexpression of HAP1 causes the formation of enlarged early endosomes, and inhibits the degradation of internalized epidermal growth factor receptors. Whereas overexpression of HAP1 does not affect either constitutive or ligand-induced receptor-mediated endocytosis, it potently blocks the trafficking of endocytosed epidermal growth factor receptors from early endosomes to late endosomes. These findings implicate, for the first time, the involvement of HAP1 in the regulation of vesicular trafficking from early endosomes to the late endocytic compartments.

3.341 The adenosine 2b receptor is required to the plasma membrane and associates with E3KARP and ezrin upon agonist stimulation
Sitaraman, S.V. et al
*J. Biol. Chem., **277(36)**, 33188-33195 (2002)*

We have previously shown that adenosine is formed in the intestinal lumen during active inflammation from neutrophil-derived 5'-AMP. Acting through the adenosine A2b receptor (A2bR), the luminally derived adenosine induces vectorial chloride secretion and a polarized secretion of interleukin-6 to the intestinal lumen. Although some G protein-coupled receptors interact with anchoring or signaling molecules, not much is known in this critical area for the A2bR. We used the model intestinal epithelial cell line, T84, and Caco2-BBE cells stably transfected with GFP-A2b receptor to study the intestinal A2bR. The A2bR is present in both the apical and basolateral membranes of intestinal epithelia. Apical or basolateral stimulation of the A2bR induces recruitment of the receptor to the plasma membrane and caveolar fractions. The A2bR co-immunoprecipitates with E3KARP and ezrin upon agonist stimulation. Ezrin interacts with E3KARP and PKA and the interaction between ezrin and E3KARP is enhanced by agonist stimulation. Our data suggest that the A2bR is recruited to the plasma membrane upon apical or basolateral agonist stimulation and interacts with E3KARP and ezrin. We speculate that such an interaction may not only anchor the A2bR to the plasma membrane but may also function to stabilize the receptor in a signaling complex in the plasma membrane.

3.342 Organization of the recveptor-kinase signaling array that regulates *Escherichia coli* chemotaxis
Levit, M.N., Grebe, T.W. and Stock, J.B.
*J. Biol. Chem., **277(39)**, 36748-36754 (2002)*

Motor behavior in prokaryotes is regulated by a phosphorelay network involving a histidine protein kinase, CheA, whose activity is controlled by a family of Type I membrane receptors. In a typical *Escherichia coli* cell, several thousand receptors are organized together with CheA and an Src homology 3-like protein, CheW, into complexes that tend to be localized at the cell poles. We found that these complexes have at least 6 receptors per CheA. CheW is not required for CheA binding to receptors, but is essential for kinase activation. The kinase activity per mole of bound CheA is proportional to the total bound CheW. Similar results were obtained with the *E. coli* serine receptor, Tsr, and the *Salmonella typhimurium* aspartate receptor, Tar. In the case of Tsr, under conditions optimal for kinase activation, the ratio of subunits in complexes is ~6 Tsr:4 CheW:1 CheA. Our results indicate that information from numerous receptors is integrated to control the activity of a relatively small number of kinase molecules.

3.343 A novel membrane protein, Ros3p, is required for phospholipid translocation across the plasma membrane in *Saccharomyces cerevisiae*
Kato, U. et al
J. Biol. Chem., ***277(40)***, *37855-37862 (2002)*

Ro09-0198 (Ro) is a tetracyclic peptide antibiotic that binds specifically to phosphatidylethanolamine (PE) and causes cytolysis. To investigate the molecular basis of transbilayer movement of PE in biological membranes, we have isolated a series of budding yeast mutants that are hypersensitive to the Ro peptide. One of the most sensitive mutants, designated *ros3* (Ro-sensitive 3), showed no significant change in the cellular phospholipid composition or in the sensitivity to amphotericin B, a sterol-binding polyene macrolide antibiotic. These results suggest that the mutation of *ros3* affects the PE organization on the plasma membrane, rather than PE synthesis or overall organization of the membrane structures. By functional complementation screening, we identified the gene *ROS3* affected in the mutant, and we showed that the hypersensitive phenotype was caused by the defective expression of the *ROS3* gene product, Ros3p, an evolutionarily conserved protein with two putative transmembrane domains. Disruption of the *ROS3* gene resulted in a marked decrease in the internalization of fluorescence-labeled analogs of PE and phosphatidylcholine, whereas the uptake of fluorescence-labeled phosphatidylserine and endocytic markers was not affected. Neither expression levels nor activities of ATP-binding cassette transporters of the *ros3*Δ cells differed from those of wild type cells, suggesting that Ros3p is not related to the multidrug resistance activities. Immunochemical analyses of the structure and subcellular localization showed that Ros3p was a glycosylated membrane protein localized in both the plasma membrane and the endoplasmic reticulum, and that a part of Ros3p was associated with the detergent-insoluble glycolipid-enriched complexes. These results indicate that Ros3p is a membrane glycoprotein that plays an important role in the phospholipid translocation across the plasma membrane.

3.344 The biofunctional activity of ubiquinone in lysosomal membranes
Nohl, H. and Gille, L.
Biogerontology, ***3***, *125-131 (2002)*

Ubiquinone is inhomogenously distributed insubcellular biomembranes. Apart frommitochondria where ubiquinone was demonstratedto exert bioenergetic and pathophysiologicalfunctions, unusually high levels of ubiquinonewere also reported to exist in Golgi vesiclesand lysosomes. In lysosomes the interiordiffers from other organelles by the low pH-value, which is important not only to arrestproteins but also to ensure optimal activity ofhydrolytic enzymes. Since redox-cycling ofubiquinone is associated with the acceptanceand release of protons, we assumed thatubiquinone is a part of a redox chaincontributing to unilateral proton distribution.A similar function of ubiquinone was earliersuggested by Crane to operate in Golgivesicles. Support for the involvement ofubiquinone in a presumed couple ofredox-carriers came from our observation thatalmost 70% of total lysosomal ubiquinone wasin the divalently reduced state. Furtherreduction was seen in the presence of externalNADH. Analysis of the components involved inthe transfer of reducing equivalents fromcytosolic NADH to ubiquinone revealed theexistence of a FAD-containingNADH-dehydrogenase. The latter was found toreduce ubiquinone by means of a b-typecytochrome. Proton translocation into theinterior was linked to the activity of thenovel lysosomal redox chain. Oxygen was foundto be the terminal electron acceptor, therebyalso regulating acidification of the lysosomalmatrix. In contrast to mitochondrialrespiration, oxygen was only trivalently reduced,giving rise to the release ofHO ●-radicals. The role of this novelproton-pumping redox chain and the significanceof the associated ROS formation has to beelucidated.

3.345 Palmitic is the main fatty acid carried by lipids of detergent-resistant membrane fractions from neural and non-neural cells
Pitto, M. et al
Neurochem. Res., ***27(7/8)***, *729-734 (2002)*

Lipids extracted from detergent-resistant membrane fractions, thought to derive from membrane domains, were analyzed for fatty acid composition. The proportion of palmitic acid in fractions isolated from neurons (cerebellar granule cells) and from neural-like cell lines (neuroblastoma-glioma NG108-15) nearly doubled (reaching about 54% of total fatty acids) with respect to cell WCL, indicating their enrichment in palmitic acid-carrying lipids. The proportion of palmitic acid in detergent-resistant fractions obtained from caveolin-transfected NG108-15 cells was comparable with that obtained from caveolin-negative cells, ruling out a specific role of this protein in recruiting palmitoylated lipid species. The enrichment in palmitic acid was remarked also in membrane fractions isolated from non-neuronal cell lines (A431) using either detergents or detergent-free techniques. Lipid fractionation and mass spectrometry experiments show that palmitic acid–rich phosphatidylcholine species are responsible of the peculiar fatty acid composition of these fractions. All together these results suggest that the enrichment in palmitic acid–rich phosphatidylcholine species is a common feature of neural and non-neural cell lines and may play a major role in the biogenesis of membrane domains.

3.346 Phosphorylation of FcγRIIA is required for the receptor-induced actin rearrangement and capping: the role of membrane rafts
Kwiatkowska, K., Frey, J. and Sobota, A.
J. Cell Sci., ***116***, *537-550 (2003)*

Activation of Fcγ receptor II (FcγRII) induces rearrangement of the actin-based cytoskeleton that serves as a driving force for FcRII-mediated phagocytosis and FcγRII capping. To get insight into the signaling events that lead to the actin reorganization we investigated the role of raft-associated Src family tyrosine kinases in capping of FcγRII in U937 cells. After crosslinking, FcγRII was found to be recruited to detergent-resistant membrane domains (DRMs), rafts, where it coexisted with Lyn kinase and underwent tyrosine phosphorylation. Lyn was displaced from DRMs under the influence of DL-α-hydroxymyristic acid and 2-bromopalmitic acid, agents blocking N-terminal myristoylation and palmitoylation of proteins, respectively, and after disruption of DRM integrity by depletion of plasma membrane cholesterol with ß-cyclodextrin. Under these conditions, phosphorylation of the crosslinked FcγRII was diminished and assembly of FcγRII caps was blocked. The similar reduction of FcγRII cap formation correlated with inhibition of receptor phosphorylation was achieved with the use of PP1 and herbimycin A, specific inhibitors of Src family tyrosine kinases. Phosphorylation of FcγRIIA expressed in BHK cells, lacking endogenous FcγRs, was abolished by substitution of tyrosine 298 by phenylalanine in the ITAM of the receptor. The mutant receptor did not undergo translocation towards cap-like structures and failed to promote the receptor-mediated spreading of the cells, as compared to BHK cells transfected with the wild-type FcγRIIA. On the basis of these data, we suggest that tyrosine phosphorylation of activated FcγRIIA by raft-residing tyrosine kinases of the Src family triggers signaling pathways that control the rearrangement of the actin cytoskeleton required for FcγRII-mediated motility.

3.347 Subcellular localization of host and viral proteins associated with tobamovirus RNA replication
Hagiwara, Y. et al
EMBO J., ***22(2)***, *344-353 (2003)*

Arabidopsis TOM1 (AtTOM1) and TOM2A (AtTOM2A) are integral membrane proteins genetically identified to be necessary for efficient intracellular multiplication of tobamoviruses. AtTOM1 interacts with the helicase domain polypeptide of tobamovirus-encoded replication proteins and with AtTOM2A, suggest-ing that both AtTOM1 and AtTOM2A are integral components of the tobamovirus replication complex. We show here that AtTOM1 and AtTOM2A proteins tagged with green fluorescent protein (GFP) are targeted to the vacuolar membrane (tonoplast)-like structures in plant cells. In subcellular fractionation analyses, GFPAtTOM2A, AtTOM2A and its tobacco homolog NtTOM2A were predominantly fractionated to low-density tonoplast-rich fractions, whereas AtTOM1–GFP, AtTOM1 and its tobacco homolog NtTOM1 were distributed mainly into the tonoplast-rich fractions and partially into higher-buoyant-density fractions con-taining membranes from several other organelles. The tobamovirus-encoded replication proteins were co-fractionated with both NtTOM1 and viral RNA-dependent RNA

polymerase activity. The replication proteins were also found in the fractions containing non-membrane-bound proteins, but neither NtTOM1 nor the polymerase activity was detected there. These observations suggest that the formation of tobamo-viral RNA replication complex occurs on TOM1-containing membranes and is facilitated by TOM2A.

3.348 Amyloidgenic processing of the Alzheimer β-amyloid precursor protein depends on lipid rafts
Ehehalt, R.E., Keller, P., Haass, C., Thiele, C. and Simons, K
*J. Cell Biol., **160(1)**, 113-123 (2003)*

Formation of senile plaques containing the ß-amyloid peptide (Aß) derived from the amyloid precursor protein (APP) is an invariant feature of Alzheimer's disease (AD). APP is cleaved either by ß-secretase or by α-secretase to initiate amyloidogenic (release of Aß) or nonamyloidogenic processing of APP, respectively. A key to understanding AD is to unravel how access of these enzymes to APP is regulated. Here, we demonstrate that lipid rafts are critically involved in regulating Aß generation. Reducing cholesterol levels in N2a cells decreased Aß production. APP and the ß-site APP cleavage enzyme (BACE1) could be induced to copatch at the plasma membrane upon cross-linking with antibodies and to segregate away from nonraft markers. Antibody cross-linking dramatically increased production of Aß in a cholesterol-dependent manner. Aß generation was dependent on endocytosis and was reduced after expression of the dynamin mutant K44A and the Rab5 GTPase-activating protein, RN-tre. This inhibition could be overcome by antibody cross-linking. These observations suggest the existence of two APP pools. Although APP inside raft clusters seems to be cleaved by ß-secretase, APP outside rafts undergoes cleavage by α-secretase. Thus, access of α- and ß-secretase to APP, and therefore Aß generation, may be determined by dynamic interactions of APP with lipid rafts.

3.349 Independent segregation of human immunodeficiency virus type I Gag protein complexes and lipid rafts
Ding, L., Derdowsky, A., Wang, J-J- and Spearman, P.
*J. Virol., **77(3)**, 1916-1926 (2003)*

Formation of human immunodeficiency virus type 1 (HIV-1) particles takes place at the plasma membrane of cells and is directed by the $Pr55^{Gag}$ polyprotein. A functional assembly domain (the M domain) within the N-terminal portion of $Pr55^{Gag}$ mediates the interaction of Gag with cellular membranes. However, the determinants that provide specificity for assembly on the plasma membrane, as opposed to intracellular membranes, have not been identified. Recently, it was reported that $Pr55^{Gag}$ interacts with lipid raft microdomains of the plasma membrane. We sought to identify the domains within $Pr55^{Gag}$ that contribute to lipid raft association of Gag. Here we demonstrate that the I domain is required for interaction with detergent-resistant membrane fractions (DRMs). Mutation of key I-domain residues or loss of myristylation abrogated the association of Gag with DRMs. Thus, the I domain and the M domain combine to mediate Gag-lipid raft interactions as defined by these biochemical criteria. However, Gag protein complexes defined by flotation studies were much denser than classical lipid rafts, failed to incorporate classical lipid raft marker proteins, and were not disrupted by cholesterol extraction. Large sheets of Gag protein were identified in DRM fractions upon examination by electron microscopy. These results indicate that HIV-1 $Pr55^{Gag}$ forms detergent-resistant complexes at the cellular periphery that are distinct from lipid raft microdomains.

3.350 A third human carnitine/organic cation transporter (*OCTN3*) as a candidate for the 5q31 Crohn's disease locus (*IBD5*)
Lamhonwah, A-M., Skaug, J., Scherer, S. and Tein, I.
*Biochem. Biophys. Res. Comm., **301**, 98-101 (2003)*

Organic cation transporters function primarily in the elimination of cationic drugs in kidney, intestine, and liver [1, 2 and 3]. The murine organic cation/carnitine (*Octn*) transporter family, *Octn1*, *Octn2*, and *Octn3* is clustered on mouse chromosome 11 (NCBI Accession No NW_00039). The human *OCTN1* and *OCTN2* orthologs map to the syntenic *IBD5* locus at 5q31 [1], which has been shown to confer susceptibility to Crohn's disease [4]. We show that the human OCTN3 protein, whose corresponding gene is not yet cloned or annotated in the human reference DNA sequence, does indeed exist and is uniquely involved in carnitine-dependent transport in peroxisomes. Its functional properties and inferred chromosomal location implicate it for involvement in Crohn's disease.

3.351 Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol
Labrecque, L. et al
*Mol. Biol. Cell, **14**, 334-347 (2003)*

The stimulation of vascular endothelial growth factor receptor-2 (VEGFR-2) by tumor-derived VEGF represents a key event in the initiation of angiogenesis. In this work, we report that VEGFR-2 is localized in endothelial caveolae, associated with caveolin-1, and that this complex is rapidly dissociated upon stimulation with VEGF. The kinetics of caveolin-1 dissociation correlated with those of VEGF-dependent VEGFR-2 tyrosine phosphorylation, suggesting that caveolin-1 acts as a negative regulator of VEGF R-2 activity. Interestingly, we observed that in an overexpression system in which VEGFR-2 is constitutively active, caveolin-1 overexpression inhibits VEGFR-2 activity but allows VEGFR-2 to undergo VEGF-dependent activation, suggesting that caveolin-1 can confer ligand dependency to a receptor system. Removal of caveolin and VEGFR-2 from caveolae by cholesterol depletion resulted in an increase in both basal and VEGF-induced phosphorylation of VEGFR-2, but led to the inhibition of VEGF-induced ERK activation and endothelial cell migration, suggesting that localization of VEGFR-2 to these domains is crucial for VEGF-mediated signaling. Dissociation of the VEGFR-2/caveolin-1 complex by VEGF or cyclodextrin led to a PP2-sensitive phosphorylation of caveolin-1 on tyrosine 14, suggesting the participation of Src family kinases in this process. Overall, these results suggest that caveolin-1 plays multiple roles in the VEGF-induced signaling cascade.

3.352 Regulation of integrin growth factor interactions in oligodendrocytes by lipid raft microdomains
Baron, W., Decker, L., Colognato, H. and ffrench-Constant, C.
*Current Biol., **13**, 151-155 (2003)*

Individual growth factors can regulate multiple aspects of behavior within a single cell during differentiation, with each signaling pathway controlled independently and also responsive to other receptors such as cell surface integrins. The mechanisms by which this is achieved remain poorly understood. Here we use myelin-forming oligodendrocytes and their precursors to examine the role of lipid rafts, cholesterol and sphingolipid-rich microdomains of the cell membrane implicated in cell signaling [1]. In these cells, the growth factor PDGF has sequential and independent roles in proliferation and survival [2and 3]. We show that the oligodendrocyte PDGFα receptor becomes sequestered in a raft compartment at the developmental stage when PDGF ceases to promote proliferation, but is now required for survival. We also show that laminin-2, which is expressed on axons in the CNS and which provides a target-dependent signal for oligodendrocyte survival by amplification of PDGFαR signaling [4] induces clustering of the laminin binding integrin α6β1 with the PDGFαR-containing lipid raft domains. This extracellular matrix-induced colocalization of integrin and growth factor receptor generates a signaling environment within the raft for survival-promoting PI3K/Akt activity. These results demonstrate novel signaling roles for lipid rafts that ensure the separation and amplification of growth factor signaling pathways during development.

3.353 SR-BI does not require raft/caveola localization for cholesteryl ester selective uptake in the human adrenal cell line NCI-H295R
Briand, O. et al
Biochim. Biophys. Acta, 163, 42-50 (2003)

Class B type I scavenger receptor (SR-BI) mediates the selective uptake of high-density lipoprotein (HDL)-derived cholesteryl esters (HDL-CE) in steroidogenic cells and hepatocytes. SR-BI is enriched in the caveolae of some cell types, genetically modified or not, and these domains have already been shown to constitute primary acceptors for HDL-CE. Nevertheless, the fate of caveola-free cell types has not yet been discussed.
NCI-H295R, a human adrenal cell line, highly active in HDL-CE uptake via SR-BI, does not display any morphologically defined caveolae and expresses caveolin at a very low level. Using two different fractionation protocols, we have shown, in this cell type, that SR-BI is homogeneously distributed along the plasma membrane and consists principally of a non-raft membrane-associated pool. Raft destabilisation and caveolin-1 displacement from plasma membrane did not modify the SR-BI-mediated HDL-CE selective uptake. Moreover, the induction of SR-BI expression that is associated with increased CE selective uptake was not associated with any modification in caveolin-1 expression or any raft-targeting mechanism of SR-BI in NCI-H295R.
In conclusion, we provide evidence that SR-BI does not require raft/caveola localisation to be implicated in CE selective uptake either in basal or in induced conditions.

3.354 The uracil transporter Fur4p associates with lipid rafts
Hearn, J.D., Lester, R.L. and Dickson, R.C.
*J. Biol. Chem., **278**, 3679-3686 (2003)*

Sphingolipids are abundant components of eucaryotic membranes, where they perform essential functions. To uncover new roles for sphingolipids, we studied *Saccharomyces cerevisiae lcb1–100* cells, which have a temperature-sensitive block in the first step in sphingolipid synthesis. We find that the level of all five species of the sphingoid long chain base intermediates is reduced 2–7-fold in cells grown at a permissive temperature, and the level of complex sphingolipids is reduced 50%. In addition, *lcb1–100* cells make no detectable phosphorylated sphingoid bases. After transfer to a restrictive temperature (a heat shock), the level of the major sphingoid bases drops rather than transiently rising, as in wild type cells. These changes affect *lcb1–100* cells in multiple ways. Basal uracil transport by Fur4p is reduced 25%, and when cells are heat-shocked, uracil transport activity falls rapidly and is not restored as it is in wild type cells. Restoration requires a functional secretory pathway and synthesis of complex sphingolipids, leading us to hypothesize that Fur4p associates with lipid rafts. The finding that Fur4p is insoluble in TritonX-100 at 4 °C and behaves like a raft-associated protein on a density gradient supports this hypothesis. Raft association may be essential for regulating breakdown of Fur4p in response to stresses and other factors that govern uracil transport activity. Our results show that long chain bases do not contribute to the inactivation of Fur4p transport activity after heat stress, but they are essential for some later, but unknown, process that leads to degradation of the protein. Further studies using *lcb1–100* cells should reveal new roles of sphingolipids in nutrient uptake and other membrane-dependent processes.

3.355 Transient mechanoactivation of neutral sphingomyelinase in caveolae to generate ceramide
Czarny, M., Liu, J., Oh, P. and Schnitzer, J.E.
*J. Biol. Chem., **278**, 4424-4430 (2003)*

The vascular endothelium acutely autoregulates blood flow *in vivo* in part through unknown mechano-sensing mechanisms. Here, we report the discovery of a new acute mechanotransduction pathway. Hemodynamic stressors from increased vascular flow and pressure *in situ* rapidly and transiently induce the activity of neutral sphingomyelinase but not that acid sphingomyelinase in a time- and flow rate-dependent manner, followed by the generation of ceramides. This acute mechanoactivation occurs directly at the luminal endothelial cell surface primarily in caveolae enriched in sphingomyelin and neutral sphingo-myelinase, but not acid sphingomyelinase. Scyphostatin, which specifically blocks neutral but not acid sphingomyelinase, inhibits mechano-induced neutral sphingomyelinase activity as well as downstream activation of extracellular signalregulated kinase 1 and 2 (ERK1 and ERK2) by increased flow *in situ*. We postulate a novel physiological function for neutral sphingomyelinase as a new mechanosensor initiating the ERK cascade and possibly other mechanotransduction pathways.

3.356 Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Groot, F.G.
J. Cell Biol., ***160(3)****, 321-328 (2003)*

The protective antigen (PA) of the anthrax toxin binds to a cell surface receptor and thereby allows lethal factor (LF) to be taken up and exert its toxic effect in the cytoplasm. Here, we report that clustering of the anthrax toxin receptor (ATR) with heptameric PA or with an antibody sandwich causes its association to specialized cholesterol and glycosphingolipid-rich microdomains of the plasma membrane (lipid rafts). We find that although endocytosis of ATR is slow, clustering it into rafts either via PA heptamerization or using an antibody sandwich is necessary and sufficient to trigger efficient internalization and allow delivery of LF to the cytoplasm. Importantly, altering raft integrity using drugs prevented LF delivery and cleavage of cytosolic MAPK kinases, suggesting that lipid rafts could be therapeutic targets for drugs against anthrax. Moreover, we show that internalization of PA is dynamin and Eps15 dependent, indicating that the clathrin-dependent pathway is the major route of anthrax toxin entry into the cell. The present work illustrates that although the physiological role of the ATR is unknown, its trafficking properties, i.e., slow endocytosis as a monomer and rapid clathrin-mediated uptake on clustering, make it an ideal anthrax toxin receptor.

3.357 APH-1 I teracts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin-nicastrin complexes

Gu, Y. et al
J. Biol. Chem., ***278(9)****, 7374-7380 (2003)*

APH-1 and PEN-2 genes modulate the function of nicastrin and the presenilins in *Caenorhabditis elegans*. Preliminary studies in transfected mammalian cells overexpressing tagged APH-1 proteins suggest that this genetic interaction is mediated by a direct physical interaction. Using the APH-1 protein encoded on human chromosome 1 (APH-1_1L; also known as APH-1a) as an archetype, we report here that endogenous forms of APH-1 are predominantly expressed in intracellular membrane compartments, including the endoplasmic reticulum and *cis*-Golgi. APH-1 proteins directly interact with immature and mature forms of the presenilins and nicastrin within high molecular weight complexes that display γ- and ε-secretase activity. Indeed APH-1 proteins can bind to the nicastrin Δ312-369 loss of function mutant, which does not undergo glycosylation maturation and is not trafficking beyond the endoplasmic reticulum. The levels of expression of endogenous APH-1_1L can be suppressed by overexpression of any other members of the APH-1 family, suggesting that their abundance is coordinately regulated. Finally, although the absence of APH-1 destabilizes the presenilins, in contrast to nicastrin and PEN-2, APH-1 itself is only modestly destabilized in cells lacking functional expression of presenilin 1 or presenilin 2. Taken together, our data suggest that APH-1 proteins, and APH-1_1 in particular, may have a role in the initial assembly and maturation of presenilin·nicastrin complexes.

3.358 Dynamin-like protein 1 is involved in peroxisomal fission

Koch, A. et al
J. Biol. Chem., ***278(10)****, 8597-8605 (2003)*

The mammalian dynamin-like protein 1 (DLP1), a member of the dynamin family of large GTPases, possesses mechanochemical properties known to constrict and tubulate membranes. In this study, we have combined two experimental approaches, induction of peroxisome proliferation by Pex11pβ and expression of dominant-negative mutants, to test whether DLP1 plays a role in peroxisomal growth and division. We were able to localize DLP1 in spots on tubular peroxisomes in HepG2 cells. In addition, immunoblot analysis revealed the presence of DLP1 in highly purified peroxisomal fractions from rat liver and an increase of DLP1 after treatment of rats with the peroxisome proliferator bezafibrate. Expression of a dominant negative DLP1 mutant deficient in GTP hydrolysis (K38A) either alone or in combination with Pex11pβ caused the appearance of tubular peroxisomes but had no influence on their intracellular distribution. In co-expressing cells, the formation of tubulo-reticular networks of peroxisomes was promoted, and peroxisomal division was completely inhibited. These findings were confirmed by silencing of DLP1 using siRNA. We propose a direct role for the dynamin-like protein DLP1 in peroxisomal fission and in the maintenance of peroxisomal morphology in mammalian cells.

3.359 Oligomeric and polymeric aggregates formed by proteins containing expanded polyglutamine
Iuchi, S., Hoffner, G., Verbeke, P., Djian, P. And Green, H.
*PNAS, **100(5)**, 2409-2414 (2003)*

Neurological diseases resulting from proteins containing expanded polyglutamine (polyQ) are characteristic-ally associated with insoluble neuronal inclusions, usually intranuclear, and neuronal death. We describe here oligomeric and polymeric aggregates formed in cells by expanded polyQ. These aggregates are not dissocia-ted by concentrated formic acid, an extremely effective solvent for otherwise insoluble proteins. Perinuclear inclusions formed in cultured cells by expanded polyQ can be completely dissolved in concentrated formic acid, but a soluble protein oligomer containing the expanded polyQ and released by the formic acid is not dissociated to monomer. In Huntington's disease, a formic acid-resistant oligomer is present in cerebral cort-ex, but not in cerebellum. Cortical nuclei contain a polymeric aggregate of expanded polyQ that is insoluble in formic acid, does not enter polyacrylamide gels, but is retained on filters. This finding shows that the process of polymerization is more advanced in the cerebral cortex than in cultured cells. The resistance of oligomer and polymer to formic acid suggests the participation of covalent bonds in their stabilization.

3.360 Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface
Paterson, R.G., Takeda, M., Ohigashi, Y., Pinto, L.H. and Lamb, R.A.
*Virology, **306**, 7-17 (2003)*

The influenza B virus BM2 protein contains 109 amino acid residues and it is translated from a bicistronic mRNA in an open reading frame that is +2 nucleotides with respect to the matrix (M1) protein. The amino acid sequence of BM2 contains a hydrophobic region (residues 7–25) that could act as a transmembrane (TM) anchor. Analysis of properties of the BM2 protein, including detergent solubility, insolubility in alkali pH 11, flotation in membrane fractions, and epitope-tagging immunocytochemistry, indicates BM2 protein is the fourth integral membrane protein encoded by influenza B virus in addition to hemagglutinin (HA), neuraminidase (NA), and the NB glycoprotein. Biochemical analysis indicates that the BM2 protein adopts an $N_{out}C_{in}$ orientation in membranes and fluorescence microscopy indicates BM2 is expressed at the cell surface. As the BM2 protein possesses only a single hydrophobic domain and lacks a cleavable signal sequ-ence, it is another example of a Type III integral membrane protein, in addition to M_2, NB, and CM2 prot-eins of influenza A, B, and C viruses, respectively. Chemical cross-linking studies indicate that the BM2 protein is oligomeric, most likely a tetramer. Comparison of the amino acid sequence of the TM domain of the BM2 protein with the sequence of the TM domain of the proton-selective ion channel M_2 protein of influenza A virus is intriguing as M_2 protein residues critical for ion selectivity/activation and channel gating (H^{37} and W^{41}, respectively) are found at the same relative position and spacing in the BM2 protein (H^{19} and W^{23}).

3.361 Mammalian Ykt6 is a neuronal SNARE targeted to a specialized compartment by its profilin-like amino terminal domain
Hasegawa, H. et al
*Mol. Biol. Cell, **14**, 698-720 (2003)*

SNAREs are required for specific membrane fusion throughout the endomembrane system. Here we report the characterization of rat ykt6, a prenylated SNARE selectively expressed in brain neurons. Immunofluor-escence microscopy in neuronal and neuroendocrine cell lines revealed that membrane-associated ykt6 did not colocalize significantly with any conventional markers of endosomes, lysosomes, or the secretory path-way. However, ykt6-containing membranes displayed very minor overlaps with lysosomes and dense-core secretory granules and were similar to lysosomes in buoyant density. Thus, ykt6 appears to be specialized for the trafficking of a unique membrane compartment, perhaps related to lysosomes, involved in aspects of neuronal function. Targeting of this SNARE to the ykt6 compartment was mediated by its profilin-like amino-terminal domain, even in the absence of protein prenylation. Although several other R-SNAREs contain related amino-terminal domains, only the ykt6 version was able to confer the specialized localiz-ation. Rat ykt6, which contains an arginine in its SNARE motif zero-layer, was found to behave like other R-SNAREs in its SNARE assembly properties. Interestingly, cytosolic ykt6, constituting more than half of the total cellular pool, appeared to be conformationally inactive for SNARE complex assembly, perhaps

indicative of a regulatory mechanism that prevents promiscuous and potentially deleterious SNARE interactions.

3.362 Inhibitors of glycosphingolipid biosynthesis reduce transepithelial electrical resistance in MDCK I and FRT cells

Leung, L.W., Contreras, R.G., Flores-Maldomado, C., Cereijido, M. and Rodriguez-Boulan, E.
*Am. J. Physiol. Cell Physiol., **284**, C1021-C1030 (2003)*

Madin-Darby canine kidney (MDCK) I and Fisher rat thyroid (FRT) cells exhibit transepithelial electrical resistance (TER) values in excess of 5,000 $\Omega \cdot cm^2$. When these cells were incubated in the presence of various inhibitors of sphingolipid biosynthesis, a >5-fold reduction of TER was observed without changes in the gate function for uncharged solutes or the fence function for apically applied fluorescent lipids. The localization of ZO-1 and occludin was not altered between control and inhibitor-treated cells, indicating that the tight junction was still intact. Furthermore, the complexity of tight junction strands, analyzed by freeze-fracture microscopy, was not reduced. Once the inhibitor was removed and the cells were allowed to synthesize sphingolipids, a gradual recovery of the TER was observed. Interestingly, these inhibitors did not attenuate the TER of MDCK II cells, a cell line that typically exhibits values below 800 $\Omega \cdot cm^{2.}$ These results suggest that glycosphingolipids play a role in regulating the electrical properties of epithelial cells.

3.363 Regulatory volume decrease in *Trypanosoma cruzi* involves amoni acid efflux and changes in intracellular calcium

Rohloff, P., Rodrigues, C.O. and Dacompo, R.
*Mol. Biochem. Parasit., **126**, 219-230 (2003)*

A regulatory volume decrease (RVD) in response to hyposmotic stress has been characterized in different life-cycle stages of *Trypanosoma cruzi*. Hyposmotic stress initially caused swelling, but this was rapidly reversed by a compensatory volume reversal that was essentially complete by 5 min. Volume recovery was associated with an amino acid efflux that accounted for approximately 50% of the regulatory volume decrease in all three life-cycle stages. The amino acid efflux was selective for neutral and anionic amino acids, but excluded cationic amino acids. Acidocalcisomes contained an amino acid pool over four times more concentrated than whole-cell levels, but about 90% of this was composed of Arg and Lys, so involvement of this pool in amino acid efflux was ruled out. Hyposmotic stress induced a rise in intracellular calcium that was dependent on influx of calcium across the plasma membrane, since chelation of extracellular calcium abolished the response. Influx of calcium was confirmed by demonstration of manganese-mediated quenching of intracellular fura-2 fluorescence and partial inhibition of the rise in calcium by calcium channel blockers. Manipulation of intra- and extracellular calcium levels had minor effects on the initial rate of amino acid efflux and no effect on the rate of volume recovery.

3.364 Caveolar compartmentation of caspase-3 in cardiac endothelial cells

Oxhorn, B.C. and Buxton, I.L.O.
*Cellular Signalling, **15**, 489-496 (2003)*

Endothelial cell apoptosis is intimately involved in the balance between blood vessel growth and regression and is promoted by numerous stimuli including angiostatin and endostatin, reactive oxygen species (ROS) released during inflammatory processes, and chronic use of drugs of abuse such as cocaine. Apoptosis is characterized by many biological signalling events, including the activation of caspases. Caveolar domains have been hypothesized to mediate apoptotic signalling. We have addressed this hypothesis in cardiac endothelial cells and here we show that caspase-3 proenzyme (32 kDa) and its activated counterpart (17 kDa) co-purify with low-density, caveolin-enriched microdomains and that caspase-3 can be localized with caveolae in intact cells using fluorescent microscopy. Disruption of caveolae results in temporal and spatial changes in enzyme activity. While caspase-3 has been associated with mitochondrial, cytosolic, and high-density regions, the co-purification of activated caspase-3 and caveolar domains reported here suggests the possibility that sarcolemmal caspase-3 may be targeted to plasma-membrane associated substrates.

3.365 Rapid localization of Gag/GagPol Complexes to detergent-resistant membrane during the assembly of human immunodeficiency virus type 1
Halwani, R., Khorchid, A., Cen, S. and Kleiman, L
*J. Virol., **77(7)**, 3973-3984 (2003)*

During human immunodeficiency virus type 1 (HIV-1) assembly in HIV-1-transfected COS7 cells, almost all steady-state Gag/Gag and Gag/GagPol complexes are membrane bound. However, exposure to 1% Triton X-100 gives results indicating that while all Gag/GagPol complexes remain associated with the detergent-resistant membrane (DRM), only 30% of Gag/Gag complexes are associated with the DRM. Analysis of the localization of newly synthesized Gag/Gag and Gag/GagPol to the membrane indicates that after a 10-min pulse with radioactive [^{35}S]Cys-[^{35}S]Met, all newly synthesized Gag/GagPol is found at the DRM. Only 30% of newly synthesized Gag/Gag moves to the membrane, and at 0 min of chase, only 38% of this membrane-bound Gag/Gag is associated with the DRM. During the first 30 min of chase, most membrane-bound Gag/Gag moves to the DRM, while between 30 and 60 min of chase, there is a significant decrease in membrane-bound Gag/Gag and Gag/GagPol. Since the localization of newly synthesized Gag/Gag to the DRM and the interaction of GagPol with Gag both depend upon Gag multimerization, the rapid localization of GagPol to the DRM probably reflects the interaction of all newly synthesized GagPol with the first newly synthesized polymeric Gag to associate with the DRM.

3.366 Localization of presenilin-nicastrin complexes and γ-secretase activity to the *trans*-Golgi network
Siman, R. and Velji, J.
*J. Neurochem., **84**, 1143-1153 (2003)*

Abundant biochemical and genetic evidence suggests that presenilins are catalytic components of γ-secretase, the protease responsible for generating the Alzheimer amyloid ß-protein. However, the differential localization of presenilins to early secretory compartments and γ-secretase substrates to late secretory compartments and the plasma membrane (the 'spatial paradox') argues against this view. We investigated this issue by studying the localization of nicastrin, another putative γ-secretase component, and its association with presenilin-1 into proteolytically active complexes. Glycosidase digests revealed that nicastrin exists in multiple glycoforms and is terminally sialylated, a modification often associated with the *trans* -Golgi network. Trafficking of nicastrin to the *trans* -Golgi network was confirmed by density gradient fractionation and immunofluorescence microscopy. In presenilin-deficient cells, however, nicastrin trafficking and maturation were abnormal, as the protein was restricted to early secretory compartments and failed to be sialylated. Mature sialylated nicastrin in *trans* -Golgi network fractions was complexed quantitatively with N- and C-terminal fragments of presenilin-1, whereas immature nicastrin present in early secretory compartments was not. Additionally, *trans* -Golgi network fractions contained the γ-secretase substrate ß-amyloid precursor protein C83 and were enriched in presenilin-dependent γ-secretase proteolytic activity. The results resolve the apparent spatial paradox by demonstrating that presenilin–nicastrin complexes and presenilin-dependent γ-secretase activity are co-localized to a late secretory compartment. The findings provide further evidence that presenilin-containing complexes are the γ-secretase, and indicate that presenilins also regulate γ-secretase assembly.

3.367 Do lipid raft mediate virus assembly and pseudotyping?
Briggs, J.A., Wilk, T. and Fuller, S.D.
*J. Gen. Virol., **84**, 757-768 (2003)*

Co-infection of a host cell by two unrelated enveloped viruses can lead to the production of pseudotypes: virions containing the genome of one virus but the envelope proteins of both viruses. The selection of components during virus assembly must therefore be flexible enough to allow the incorporation of unrelated viral membrane proteins, yet specific enough to exclude the bulk of host proteins. This apparent contradiction has been termed the pseudotypic paradox. There is mounting evidence that lipid rafts play a role in the assembly pathway of non-icosahedral, enveloped viruses. Viral components are concentrated initially in localized regions of the plasma membrane via their interaction with lipid raft domains. Lateral interactions of viral structural proteins amplify the changes in local lipid composition which in turn enhance the concentration of viral proteins in the rafts. An affinity for lipid rafts may be the common feature of enveloped virus proteins that leads to the formation of pseudotypes.

3.368 Mastoparan selectively activates phospholipase D2 in cell membranes
Chahdi, A., Choi, W.S., Kim, Y. M. and Beaven, M.A.

J. Biol. Chem., ***278(14)***, *12039-12045 (2003)*

Both known isoforms of phospholipase (PL) D, PLD1 and PLD2, require phosphatidylinositol 4,5-bisphosphate for activity. However, PLD2 is fully active in the presence of this phospholipid, whereas PLD1 activation is dependent on additional factors such as ADP-ribosylation factor-1 (ARF-1) and protein kinase Cα. We find that mastoparan, an activator of G_i and mast cells, stimulates an intrinsic PLD activity, most likely PLD2, in fractions enriched in plasma membranes from rat basophilic leukemia 2H3 mast cells. Overexpression of PLD2, but not of PLD1, results in a large increase in the mastoparan-inducible PLD activity in membrane fractions, particularly those enriched in plasma membranes. As in previous studies, expressed PLD2 is localized primarily in the plasma membrane and PLD1 in granule membranes. Studies with pertussis toxin and other agents indicate that mastoparan stimulates PLD2 independently of G_i, ARF-1, protein kinase C, and calcium. Kinetic studies indicate that mastoparan interacts synergistically with phosphatidylinositol 4,5-bisphosphate and that oleate, itself a weak stimulant of PLD2 at low concentrations, is a competitive inhibitor of mastoparan stimulation of PLD2. Therefore, mastoparan may be useful for investigating the regulation of PLD2, particularly in view of the well studied molecular interactions of mastoparan with certain other strategic signaling proteins.

3.369 Human immunodeficiency virus type 1 assembly and lipid rafts: Pr55^{gag} associates with membrane domains

Holm, K., Weclewicz,K., Hewson, R. and Suomalainen, M.
*J. Virol.,**77**, 4805-4817 (2003)*

The assembly and budding of human immunodeficiency virus type 1 (HIV-1) at the plasma membrane are directed by the viral core protein Pr55^{gag}. We have analyzed whether Pr55^{gag} has intrinsic affinity for sphingolipid- and cholesterol-enriched raft microdomains at the plasma membrane. Pr55^{gag} has previously been reported to associate with Triton X-100-resistant rafts, since both intracellular membranes and virus-like Pr55^{gag} particles (VLPs) yield buoyant Pr55^{gag} complexes upon Triton X-100 extraction at cold temperatures, a phenotype that is usually considered to indicate association of a protein with rafts. However, we show here that the buoyant density of Triton X-100-treated Pr55^{gag} complexes cannot be taken as a proof for raft association of Pr55^{gag}, since lipid analyses of Triton X-100-treated VLPs demonstrated that the detergent readily solubilizes the bulk of membrane lipids from Pr55^{gag}. However, Pr55^{gag} might nevertheless be a raft-associated protein, since confocal fluorescence microscopy indicated that coalescence of GM1-positive rafts at the cell surface led to copatching of membrane-bound Pr55^{gag}. Furthermore, extraction of intracellular membranes or VLPs with Brij98 yielded buoyant Pr55^{gag} complexes of low density. Lipid analyses of Brij98-treated VLPs suggested that a large fraction of the envelope cholesterol and phospholipids was resistant to Brij98. Collectively, these results suggest that Pr55^{gag} localizes to membrane microdomains that are largely resistant to Brij98 but sensitive to Triton X-100, and these membrane domains provide the platform for assembly and budding of Pr55^{gag} VLPs.

3.370 Regulation of cytochrome c oxidase activity by c-Src in osteoclasts

Miyazaki, T., Neff, L., Tanaka, S., Horne, W.C. and Baron, R.
J. Cell Biol., ***160(5)***, *709-718 (2003)*

The function of the nonreceptor tyrosine kinase c-Src as a plasma membrane–associated molecular effector of a variety of extracellular stimuli is well known. Here, we show that c-Src is also present within mitochondria, where it phosphorylates cytochrome *c* oxidase (Cox). Deleting the c-*src* gene reduces Cox activity, and this inhibitory effect is restored by expressing exogenous c-Src. Furthermore, reducing endogenous Src kinase activity down-regulates Cox activity, whereas activating Src has the opposite effect. Src-induced Cox activity is required for normal function of cells that require high levels of ATP, such as mitochondria-rich osteoclasts. The peptide hormone calcitonin, which inhibits osteoclast function, also down-regulates Cox activity. Increasing Src kinase activity prevented the inhibitory effect of calcitonin on Cox activity and osteoclast function. These results suggest that c-Src plays a previously unrecognized role in maintaining cellular energy stores by activating Cox in mitochondria.

3.371 Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system
McClelland, G.B., Khanna, S., Gonzales, G.F., Butz, C.E. and Brooks, G.A.
*Biochem. Biophys. Res. Comm., **304**, 130-135 (2003)*

One of the many functions of liver peroxisomes is the β-oxidation of long-chain fatty acids. It is essential for the continuation of peroxisomal β-oxidation that a redox shuttle system exist across the peroxisomal membrane to reoxidize NADH. We propose that this redox shuttle system consists of a substrate cycle between lactate and pyruvate. Here we present evidence that purified peroxisomal membranes contain both monocarboxylate transporter 1 (MCT 1) and MCT 2 and that along with peroxisomal lactate dehydrogenase (pLDH) form a Peroxisomal Lactate Shuttle. Peroxisomal β-oxidation was greatly stimulated by the addition of pyruvate and this increase was partially inhibited by the addition of the MCT blocker α-cyano-4-hydroxycinnamate (CINN). We also found that peroxisomes generated lactate in the presence of pyruvate. Together these data provide compelling that the Peroxisome Lactate Shuttle helps maintain organelle redox and the proper functioning of peroxisomal β-oxidation.

3.372 Modulation of Rho GTPase signaling regulates a switch between adigenesis and myogenesis
Sordella, R. et al
*Cell, **113**, 147-158 82003)*

Mature adipocytes and myocytes are derived from a common mesenchymal precursor. While IGF-1 promotes the differentiation of both cell types, the signaling pathways that specify the distinct cell fates are largely unknown. Here, we show that the Rho GTPase and its regulator, p190-B RhoGAP, are components of a critical switch in the adipogenesis-myogenesis "decision." Cells derived from embryos lacking p190-B RhoGAP exhibit excessive Rho activity, are defective for adipogenesis, but undergo myogenesis in response to IGF-1 exposure. In vitro, activation of Rho-kinase by Rho inhibits adipogenesis and is required for myogenesis. The activation state of Rho following IGF-1 signaling is determined by the tyrosine-phosphorylation status of p190-B RhoGAP and its resulting subcellular relocalization. Moreover, adjusting Rho activity is sufficient to alter the differentiation program of adipocyte and myocyte precursors. Together, these results identify the Rho GTPase as an essential modulator of IGF-1 signals that direct the adipogenesis-myogenesis cell fate decision.

3.373 The SNARE motif contributes to rbet1 intracellular targeting and dynamics independently of SNARE interactions
Joglekar, A.P., Xu, D., Rigotti, D.J., Fairman, R. and Hay, J.C.
*J. Biol. Chem., **278(16)**, 14121-14133 (2003)*

The endoplasmic reticulum/Golgi SNARE rbet1 cycles between the endoplasmic reticulum and Golgi and is essential for cargo transport in the secretory pathway. Although the quaternary SNARE complex containing rbet1 is known to function in membrane fusion, the structural role of rbet1 is unclear. Furthermore, the structural determinants for rbet1 targeting and its cyclical itinerary have not been investigated. We utilized protein interaction assays to demonstrate that the rbet1 SNARE motif plays a structural role similar to the carboxyl-terminal helix of SNAP-25 in the synaptic SNARE complex and demonstrated the importance to SNARE complex assembly of a conserved salt bridge between rbet1 and sec22b. We also examined the potential role of the rbet1 SNARE motif and SNARE interactions in rbet1 localization and dynamics. We found that, in contrast to what has been observed for syntaxin 5, the rbet1 SNARE motif was essential for proper targeting. To test whether SNARE interactions were important for the targeting function of the SNARE motif, we used charge repulsion mutations at the conserved salt bridge position that rendered rbet1 defective for binary, ternary, and quaternary SNARE interactions. We found that heteromeric SNARE interactions are not required at any step in rbet1 targeting or dynamics. Furthermore, the heteromeric state of the SNARE motif does not influence its interaction with the COPI coat or efficient recruitment onto transport vesicles. We conclude that protein targeting is a completely independent function of the rbet1 SNARE motif, which is capable of distinct classes of protein interactions.

3.374 Effect of glycosylphosphatidylinositol anchor-dependent and -independent prion protein association with model raft membranes on conversion to the protease-resistant isoform

Baron, G.S. and Caughey, B.

J. Biol. Chem., ***278(17)***, *14883-14892 (2003)*

Prion protein (PrP) is usually bound to membranes by a glycosylphosphatidylinositol (GPI) anchor that associates with detergent-resistant membranes, or rafts. To examine the effect of membrane association on the interaction between the normal protease-sensitive PrP isoform (PrP-sen) and the protease-resistant isoform (PrP-res), a model system was employed using PrP-sen reconstituted into sphingolipid-cholesterol-rich raft-like liposomes (SCRLs). Both full-length (GPI^+) and GPI anchor-deficient (GPI^-) PrP-sen produced in fibroblasts stably associated with SCRLs. The latter, alternative mode of membrane association was not detectably altered by glycosylation and was markedly reduced by deletion of residues 34-94. The SCRL-associated PrP molecules were not removed by treatments with either high salt or carbonate buffer. However, only GPI^+ PrP-sen resisted extraction with cold Triton X-100. PrP-sen association with SCRLs was pH-independent. PrP-sen was also one of a small subset of phosphatidylinositol-specific phospholipase C (PI-PLC)-released proteins from fibroblast cells found to bind SCRLs. A cell-free conversion assay was used to measure the interaction of SCRL-bound PrP-sen with exogenous PrP-res as contained in microsomes. SCRL-bound GPI^+ PrP-sen was not converted to PrP-res until PI-PLC was added to the reaction or the combined membrane fractions were treated with the membrane-fusing agent polyethylene glycol (PEG). In contrast, SCRL-bound GPI^- PrP-sen was converted to PrP-res without PI-PLC or PEG treatment. Thus, of the two forms of raft membrane association by PrP-sen, only the GPI anchor-directed form resists conversion induced by exogenous PrP-res.

3.375 Plasma membrane rafts of rainbow trout are subject to thermal acclimation

Zehmer, J.K. and Hazel, J.R.

J. Exp. Biol., ***206***, *1657-1667 (2003)*

Rafts are cholesterol- and sphingolipid-enriched microdomains of the plasma membrane (PM) that organize many signal transduction pathways. Interactions between cholesterol and saturated lipids lead to patches of liquid-ordered membrane (rafts) phase-separating from the remaining PM. Phase behavior is temperature sensitive, and acute changes in temperature experienced by poikilotherms would be expected to perturb raft structure, necessitating an acclimatory response. Therefore, with thermal acclimation, we would expect compositional changes in the raft directed to offset this perturbation. Using differential and density gradient centrifugation, we separated PM from the livers of rainbow trout acclimated to 5°C and 20°C into raft-enriched (raft) and raft-depleted PM (RDPM). Compared with RDPM, the raft fractions were enriched in cholesterol, the $ß_2$-adrenergic receptor and adenylyl cyclase, which are commonly used markers for this microdomain. Furthermore, cholesterol was enriched in all fractions from warm-compared with cold-acclimated animals, but this increase was 3.4 times greater in raft than in PM. We developed a novel approach for measuring membrane molecular interaction strength (and thus the tendency to stabilize raft structure) based on the susceptibility of membranes to detergent. Specifically, studies with model vesicles demonstrated that the capacity of a membrane to accommodate detergent prior to solubilization (saturation point) was a good index of this property. The saturation point of the isolated membrane preparations was temperature sensitive and was significantly different in 5°C- and 20°C-acclimated RDPM when assayed at 5°C and 20°C, respectively. By contrast, this comparison in rafts was not significantly different, suggesting compensation of this property. These data suggest that compositional changes made in the PM during thermal acclimation act to offset thermal perturbation of the raft but not the RDPM structural integrity.

3.376 **Ligand-dependent recruitment of the ErbB4 signaling complex into neuronal lipid rafts**
Li Ma et al
J. Neurosci., ***23(8)***, *3164-3175 (2003)*

Neuregulin (NRG) regulates synapse formation and synaptic plasticity, but little is known about the regulation of NRG signaling at synapses. Here we show that the NRG receptor ErbB4 was localized in anatomically defined postsynaptic densities in the brain. In cultured cortical neurons, ErbB4 was recruited to the neuronal lipid raft fraction after stimulation by NRG. Along with ErbB4, adaptor proteins Grb2 and Shc were translocated to lipid rafts by NRG stimulation. In transfected human embryonic kidney 293 cells, the partitioning of ErbB4 into a detergent-insoluble fraction that includes lipid rafts was increased by PSD-95 (postsynaptic density-95), through interaction of the ErbB4 C terminus with the PDZ [PSD-95/Discs large/zona occludens-1] domains of PSD-95. Disruption of lipid rafts inhibited NRG-induced activation of Erk and prevented NRG-induced blockade of induction of long-term potentiation at hippocampal CA1 synapses. Thus, our results indicate that NRG stimulation causes translocation of ErbB4 into lipid rafts and that lipid rafts are necessary for signaling by ErbB4.

3.377 **Raft partitioning of the yeast uracil permease during trafficking along the endocytic pathway**
Dupre, S. and Haguenauer-Tsapis, R.
Traffic, ***4***, *83-96 (2003)*

Lipid rafts, formed by the lateral association of sphingolipids and cholesterol in the external membrane leaflet, have been implicated in membrane traffic and cell signaling in mammalian cells. Yeast plasma membranes were also recently shown to contain lipid raft microdomains consisting of sphingolipids and ergosterol, and containing several plasma membrane proteins, including Gas1p, a GPI-anchored protein, and the [H^+] ATPase Pma1p. In this study, we investigated whether lipid rafts were involved in the intracellular trafficking of a yeast transporter, uracil permease, which undergoes ubiquitin-dependent endocytosis. Regardless of its ubiquitination status, uracil permease was found to be associated with rafts in the plasma membrane. The expression of Fur4p in *lcb1-100* cells, deficient in the first enzyme of sphingolipid synthesis, impaired the association of Fur4p with detergent-resistant fractions. When targeted to endocytic compartments, uracil permease appeared to be progressively transferred to detergent-soluble fractions, suggesting that the lipid environment might change between plasma membrane and endosomes. Consistent with this hypothesis, the wild-type form of the v-SNARE Snc1p, which is known to cycle between the plasma membrane and endosomal compartments, was recovered in both detergent-resistant and detergent-soluble fractions. In contrast, a variant Snc1p that accumulates at the plasma membrane was recovered exclusively in detergent-resistant fractions.

3.378 **The structure of murine outer segment disk membranes using atomic force microscopy**
Saperstein, D.A. et al
Invest. Ophthalmol Vis. Sci., ***44***, *E-abstract 3175 (2003)*

Purpose: Atomic force microscopy (AFM) is a powerful new tool to study biologic membranes. We used AFM to study the surface of murine rod outer segment disk membranes
Methods: Dark adapted wildtype C57BL/6 mice were sacrificed and their retinas were removed. Osmotically intact rod outer segments (ROS) were isolated via centrifugation in an **Optiprep** gradient. The ROS were then burst using 2mM Tris-HCl, pH 7.4, at 0°C for 15 hr and isolated using centrifugation in an **Optiprep** gradient. The isolated disks were adsorbed to mica and scanned using a Nanoscope Multimode microscope (Digital Instruments) equipped with an infrared laser head, fluid cell, and oxide-sharpened silicon nitride cantilevers (OMCL-TR400PSA, Olympus) in aqueous fluid using the contact scanning mode. All procedures were carried out in complete darkness with the aid of night vision goggles. The disk integrity was verified by scanning and transmission electron microscopy.
Results: The superstructure of the disk membrane was revealed. The cytoplasmic surface of the disks are textured and under high magnification consist of rows of rhodopsin pairs densely packed in paracrystalline arrays. The density of rhodopsin monomers averages 48,300 molecules per μm^2. The distance measured between rhodopsin molecules in the dimer was 3.8 nm (N=40). This measurement was consistent with measurements using the angularly averaged powder diffraction pattern.
Conclusions: This study represents the first description of the higher order structure of rhodopsin molecules within the native disk membranes. The resolution is sufficient to visualize individual unstained rhodpsin molecules. The dimeric nature of rhodopsin in the disk membrane is clearly demonstrated supporting published pharmacological and biochemical analyses.

3.379 Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication
Elazar, M. et al
*J. Virol., **77(10)**, 6055-6061 (2003)*

We identified an N-terminal amphipathic helix (AH) in one of hepatitis C virus (HCV)'s nonstructural proteins, NS5A. This AH is necessary and sufficient for membrane localization and is conserved across isolates. Genetically disrupting the AH impairs HCV replication. Moreover, an AH peptide-mimic inhibits the membrane association of NS5A in a dose-dependent manner. These results have exciting implications for the HCV life cycle and novel antiviral strategies.

3.380 Role of caveolae in signal-tranducing function of cardiac Na^+/K^+ -ATPase
Liu, L. et al
*Am. J. Physiol. Cell Physiol., **284**, C1550-C1560 (2003)*

Ouabain binding to Na^+/K^+-ATPase activates Src/epidermal growth factor receptor (EGFR) to initiate multiple signal pathways that regulate growth. In cardiac myocytes and the intact heart, the early ouabain-induced pathways that cause rapid activations of ERK1/2 also regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and contractility. The goal of this study was to explore the role of caveolae in these early signaling events. Subunits of Na^+/K^+-ATPase were detected by immunoblot analysis in caveolae isolated from cardiac myocytes, cardiac ventricles, kidney cell lines, and kidney outer medulla by established detergent-free procedures. Isolated rat cardiac caveolae contained Src, EGFR, ERK1/2, and 20-30% of cellular contents of α_1- and α_2-isoforms of Na^+/K^+-ATPase, along with nearly all of cellular caveolin-3. Immunofluorescence microscopy of adult cardiac myocytes showed the presence of caveolin-3 and α-isoforms in peripheral sarcolemma and T tubules and suggested their partial colocalization. Exposure of contracting isolated rat hearts to a positive inotropic dose of ouabain and analysis of isolated cardiac caveolae showed that ouabain caused *1*) no change in total caveolar ERK1/2, but a two- to threefold increase in caveolar phosphorylated/activated ERK1/2; *2*) no change in caveolar α_1-isoform and caveolin-3; and *3*) 50-60% increases in caveolar Src and α_2-isoform. These findings, in conjunction with previous observations, show that components of the pathways that link Na^+/K^+-ATPase to ERK1/2 and $[Ca^{2+}]_i$ are organized within cardiac caveolae microdomains. They also suggest that ouabain-induced recruitments of Src and α_2-isoform to caveolae are involved in the manifestation of the positive inotropic effect of ouabain.

3.381 Resistance of cell membranes to different antigens
Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A. and Simons, K.
*Proc. Natl. Acad. Sci. USA, **100**, 5795-5800 (2003)*

Partial resistance of cell membranes to solubilization with mild detergents and the analysis of isolated detergent-resistant membranes (DRMs) have been used operationally to define membrane domains. Given the multitude of detergents used for this purpose. we sought to investigate whether extraction with different deter gents might reflect the same underlying principle of domain formation. We therefore compared the protein and lipid content of DRM5 prepared with a variety of detergents from two cell lines. We found that the detergents differ considerably in their ability to selectively solubilize membrane proteins and to enrich sphingolipids and cholesterol over glycerophospholipids as well as saturated over unsaturated phosphatidyicholine. In addition, we observed cell type-dependent variations of the molecular characteristics of DRMs and the effectiveness of particular detergents. These results make it unlikely that different detergents reflect the same aspects of membrane organization and underscore both the structural complexity of cell membranes and the need for more sophisticated analytical tools to understand their architecture.

3.382 Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form *cis* hetero-oligomers

Barletta, G-M., Kovari, I.A., Verma, R.K., Kerjaschki, D. and Holzmann, L.B.
J. Biol. Chem., ***278***, *19266-19271 (2003)*

Glomerular visceral epithelial cells (podocytes) appear to play a central role in maintaining the selective filtration barrier of the renal glomerulus. While the immunoglobulin superfamily member Nephrin was proposed to act as a cell adhesion molecule at the podocyte intercellular junction necessary for maintaining gbmerular perm selectivity, the Nephrin ligand has not been identified. The existence of a new subfamily of Nephrin-like molecules including Neph1 was recently described. Genetic deletion of Nephrin or Neph1 resulted in similar phenotypes of podocyte foot process effacement and proteinuria. The subcellular localization of Neph1 and the possibility that Nephrin and Neph1 interact was investigated. Polyclonal antiserum for Neph1 was raised and characterized. Neph1 migrated as a 90-kDa protein on SDS-PAGE under reducing conditions. Neph1 was identified in a glomerular and podocyte-specific distribution in adult rat kidney. Like Nephrin and Podocin, Neph1 was enriched in Triton X-100 detergent-resistant membrane fractions. Consistent with this observation, immunogold electron microscopy demonstrated that Neph1 localized exclusively to lateral margins of podocyte foot processes at the insertion of the slit diaphragm. Neph1 and Nephrin participate in a direct cis-interaction involving their cytoplasmic domains. In addition, interactions between the extracellular domain of Nephrin and itself and between the extracellular domain of Nephrin and that of Neph1 were detected. Neph1 did not interact via a homophilic interaction. These observations suggest that Nephrin and Neph1 form a hetero-oligomeric receptor complex in the plane of the membrane that might interact across the foot process intercellular junction through interactions between Nephrin with itself and Neph1.

3.383 Distinct rates of palmitate turnover on membrane-bound cellular and oncogenic H-Ras

Baker, T.L., Zheng, H., Walker, J., Coloff, J.L. and Buss, J.E.
J. Biol. Chem., ***278***, *19292-19300 (2003)*

H-Ras displays dynamic cycles of GTP binding and palmitate turnover. GTP binding is clearly coupled to activation, but whether the palmitoylated COOH terminus participates in signaling, especially when constrained by membrane tethering, is unknown. As a way to compare COO!! termini of membrane-bound, lipid-modified H-Ras, palmitate removal rates were measured for various forms of H-Ras in NIH 3T3 cells. Depalmitoylation occurred slowly (t_2 ~2.4 h) in cellular (H-RasWT) or dominant negative (H-Rasl7N) forms and more rapidly (t_2 ~1 h) in oncogenic H-Ras6lL or H-RasRl2,T59. Combining this data with GTP binding measurements, the palmitate half-life of H-Ras in the fully GTP-bound state was estimated to be less than 10 min. Slow palmitate removal from cellular H-Ras was not explained by sequestration in caveolae, as neither cellular nor oncogenic H-Ras showed alignment with caveolin by immunofluorescence. Conversely, although it had faster palmitate removal, oncogenic H-Ras was located in the same fractions as H-RasWT on four types of density gradients, and remained fully membrane-bound. Thus the different rates of deacylation occurred even though oncogenic and cellular H-Has appeared to be in similar locations. Instead, these results suggest that acyiprotein thioesterases access oncogenic H-Ras more easily because the conformation of its COOH terminus against the membrane is altered. This previously undetected difference could help produce distinctive effector interactions and signaling of oncogenic H-Ras.

3.384 Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function: I Mutation of amino-terminalcysteine residues on RGS16 prevents its targeting to lipid rafts and palmitoylation of an internal cysteine residue.

Hiol, A et al

J. Biol. Chem., ***278***, *19301-19308 (2003)*

Regulators of G-protein signaling (RGS) proteins down-regulate signaling by heterotrimeric G-proteins by accelerating GTP hydrolysis on the Gα subunits. Palmitoylation, the reversible addition of palmitate to cysteine residues, occurs on several RGS proteins and is critical for their activity. For RGS16, mutation of Cys-2 and Cys-12 blocks its incorporation of E^3Hlpalmitate and ability to turn-off G_i and G_q signaling and significantly inhibited its GTPase activating protein activity toward a Gα subunit fused to the 5-hydroxytryptamine receptor 1A, but did not reduce its plasma membrane localization based on cell fractionation studies and immunoelectron microscopy. Palmitoylation can target proteins, including many signaling proteins, to membrane microdomains, called lipid rafts. A subpopulation of endogenous RGS16 in rat liver membranes and overexpressed RGS16 in COS cells, but not the non-palmitoylated cysteine mutant of RGS16, localized to lipid rafts. However, disruption of lipid rafts by treatment with methyl-j3-cyclodextrin did not decrease the GTPase activating protein activity of RGS16. The lipid raft fractions were enriched in protein acyltransferase activity, and RGS16 incorporated E^3Hlpalmitate into a peptide fragment containing Cys-98, a highly conserved cysteine within the RGS box. These results suggest that the amino-terminal palmitoylation of an RGS protein promotes its lipid raft targeting that allows palmitoylation of a poorly accessible cysteine residue that we show in the accompanying article (Osterhout, J. L., Waheed, A. A., Hiol, A., Ward, R. J., Davey, P. C., Nini, L., Wang, J., Milligan, G., Jones, T. L. Z., and Druey, K. M. (2003) *J. BioL Chem.* 278,19309-19316) was critical for RGS16 and RGS4 GAP activity.

3.385 Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function: II Palmitoylation of a cysteine residue in the RGS box is critical for RGS16 GTPase accelerating activity and regulation of G_i-coupled signaling

Osterhout, J.L. et al.

J. Biol. Chem., ***278***, *19309-19316 (2003)*

Palmitoylation is a reversible post-translational modification used by cells to regulate protein activity. The regulator of G-protein signaling (RGS) proteins RGS4 and RGS16 share conserved cysteine (Cys) residues that undergo palmitoylation. In the accompanying article (Hiol, A., Davey, P. C., Osterhout, J. L., Waheed, A. A., Fischer, E. H., Chen, C. K., Milligan, G., Druey, K. M., and Jones, T. L. Z. (2003) *J. BioL Chem.* 278, 19301-19308), we determined that mutation of NH_2-terminal cysteine residues in RGS16 (Cys-2 and Cys-12) reduced GTPase accelerating (GAP) activity toward a 5-hydroxytryptamine ($5\text{-}HT_{1A}$)/$G\alpha_{o1}$ receptor fusion protein in cell membranes. NH_2-terminal acylation also permitted palmitoylation of a cysteine residue in the RGS box of RGS16 (Cys-98). Here we investigated the role of internal palmitoylation in RGS16 localization and GAP activity. Mutation of RGS16 Cys.98 or RGS4 Cys-95 to alanine reduced GAP activity on the $5\text{-}HT_{1A}$/$G\alpha_{o1}$ fusion protein and regulation of adenylyl cyclase inhibition. The C98A mutation had no effect on RGS16 localization or GAP activity toward purified G-protein a subunits. Enzymatic palmitoylation of RGS16 resulted in internal palmitoylation on residue Cys-98. Palmitoylated RGS16 or RGS4 WT but not C98A or C95A preincubated with membranes expressing $5\text{-}HT_{1A}$/$G\alpha_{o1}$ displayed increased GAP activity over time. These results suggest that palmitoylation of a Cys residue in the RGS box is critical for RGS16 and RGS4 GAP activity and their ability to regulate G_i-coupled signaling in mammalian cells.

3.386 Differential mobilization of newly synthesized cholesterol and biosynthetic sterol precursors from cells

Lusa, S., Heino, S. and Ikonen, E.
J. Biol. Chem., ***278***, *19844-19851 (2003)*

Previous work demonstrates that the biosynthetic precursor of cholesterol, desmosterol, is released from cells and that its efflux to high density lipoprotein or phosphatidylcholine vesicles is greater than that of newly synthesized cholesterol (Johnson, W. J., Fischer, H. T., Phillips, M. C., and Rothblat, G. H. (1995) *J. BioL Chem.* 270, 25037-25046). Here we report that the release of individual precursor sterols varies with the efflux of newly synthesized zymosterol being greater than that of lathosterol and both exceeding that of newly synthesized cholesterol when using either methyl-β-cyclodex-trin or complete serum as acceptors. The transfer of newly synthesized lathosterol to methyl-β-cyclodextrin was inhibited by actin polymerization but not by Golgi disassembly whereas that of newly synthesized cholesterol was inhibited by both conditions. Newly synthesized lathosterol associated with cellular detergent-resistant membranes more rapidly than newly synthesized cholesterol. Upon efflux to serum, newly synthesized cholesterol precursors associated with both high and low density lipoproteins. Stimulation of the formation of direct endoplasmic reticulum-plasma membrane contacts was accompanied by enhanced efflux of newly synthesized lathosterol but not of newly synthesized cholesterol to serum acceptors. The data indicate that the efflux of cholesterol precursors differs not only from that of cholesterol but also from each other, with the more polar zymosterol being more avidly effluxed. Moreover, the results suggest that the intracellular routing of cholesterol precursors differs from that of newly synthesized cholesterol and implicates a potential role for the actin cytoskeleton and endoplasmic reticulum-plasma membrane contacts in the efflux of lathosterol.

3.387 Presenilin 1 and Presenilin 2 have differential effects on the stability and maturation of Nicastrin in mammalian brain

Chen, F. et al.
J. Biol. Chem., ***278***, *19974-19979 (2003)*

The presenilins and nicastrin form high molecular mass, multimeric protein complexes involved in the in. tramembranous proteolysis of several proteins. Post-translational glycosylation and trafficking of nicastrin is necessary for the activity of these complexes. We report here that although there are differences in the post-translational processing of nicastrin in neurons and glia, both of the presenilins are required for the physiological post-translational modification and for the correct subcellular distribution of nicastrin. Absence of presenilin 1 (PS1) is associated with dramatic reductions in the level of mature glycosylated nicastrin and with redistribution of nicastrin away from the cell surface. In contrast, absence of presenilin 2 (PS2) is associated with only modest reductions in the levels of immature nicastrin. It is notable that these differential effects parallel the differential effects of null mutations in PS1 and PS2 on APP and Notch processing. Our data there. fore suggest that the differential interactions of PS1 and PS2 with nicastrin reflect different functions for the PS1 and PS2 complexes.

3.388 Synergistic assembly of linker for activation of T cells signaling protein complexes in T cell plasma membrane domains

Hartgrove, L., Lin, J., Langen, H., Zech, T., Weiss, A. and Harder, T.
J. Biol. Chem., ***278***, *20389-20394 (2003)*

Transmembrane adaptor molecule LAT (linker for activation of T cells) forms a central scaffold for signaling protein complexes that accumulate in the vicinity of activated T cell antigen receptors (TCR). Here we used biochemical analysis of immunoisolated plasma membrane domains and fluorescence imaging of green fluorescence protein-tagged signaling proteins to investigate the contributions of different tyrosine.based signaling protein docking sites of LAT to the formation of LAT signaling protein assemblies in TCR membrane domains. We found that the phospholipase C γ docking site of LAT and different Grb2/Gads docking sites function in an interdependent fashion and synergize to accumulate LAT, Grb2, and phospholipase C γ in TCR signaling assemblies. Two-dimensional gels showed that Grb2 is a predominant cytoplasmic adaptor in the isolated LAT signaling complexes, whereas Gads, Crk-1, and Grap are present in lower amounts. Taken together our data suggest a synergistic assembly of multimolecular TCR-LAT signal transduction complexes in T cell plasma membrane domains.

3.389 A novel method of imaging lysosomes in living human mammary epithelial cells

Glunde, K., Guggino, S.E., Ichikawa, Y. and Bhujwalla, Z.M.
Mol. Imaging, ***2***, *24-36 (2003)*

Cancer cells Invade by secreting degradative enzymes which, under normal conditions, are sequestered in lysosomal vesicles. The ability to non-invasively label lysosomes and track lysosomal trafficking would be extremely useful to understand the mechanisms by which degradative enzymes are secreted in the presence of pathophysiological environments, such as hypoxia and acidic extracellular pH, which are frequently encountered in solid tumors. In this study, a novel method of introducing a fluorescent label into lysosomes of human mammary epithellal cells (HMECs) was evaluated. Highly glycosylated lysosomal membrane proteins were labeled with a newly synthesized compound, 5-dimethylamino-naphthalene-1-sulfonic acid 5-amino-3,4,6-trihydroxy-tetrahydro-pyran-2-ylmethyl ester (6-O-dansyl.G1cNH_2). The ability to optically image lysosomes using this new probe was validated by determining the colocalization of the fluorescence from the dansyl group with immunofluorescent staining of two well-established lysosomal marker proteins, LAMP-1 and LAMP-2. The location of the dansyl group in lysosomes was also verified by using an anti-dansyl antibody in Western blots of lysosomes isolated using isopycnic density gradient centrifugation. This novel method of labeling lysosomes biosynthetically was used to image lysosomes in living HMECs perfused In a microscopy-compatible cell perfusion system.

3.390 Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin

Verma, R. et al.
J. Biol. Chem., ***278***, *20716-20723 (2003)*

Recent investigations have focused on characterizing the molecular components of the podocyte intercellular junction, because several of these components, including Nephrin, are functionally necessary for develop. ment of normal podocyte structure and filter integrity. Accumulating evidence suggests that the Nephrin-asso. ciated protein complex is a signaling nexus. As such, Nephrin-dependent signaling might be mediated in part through Nephrin phosphorylation. Described are biochemical and mouse genetics experiments demonstrating that membrane-associated Nephrin is tyrosine-phosphorylated by the Src family kinase Fyn. Nephrin fractionated in detergent-resistant glomerular membrane fractions with Fyn and Yes. Fyn directly bound Nephrin via its SH3 domain, and Fyn directly phosphorylated Nephrin. Glomeruli in which Fyn, Yes, or Fyn and Yes were genetically deleted in mice were charac. terized to explore the relationship between these kinases and Nephrin. Fyn deletion resulted in coarsening of podocyte foot processes and marked attenuation of Nephrin phosphorylation in isolated glomerular deter-gent-resistant membrane fractions. Yes deletion had no identifiable effect on podocyte morphology but dramatically increased Nephrin phosphorylating activity. Similar to Fyn deletion, simultaneous deletion of Fyn and Yes reduced Nephrin phosphorylating activity. These results demonstrate that endogenous Fyn catalyzes Nephrin phosphorylation in podocyte detergent-resistant membrane fractions. Although Yes appears to effect the regulation of Nephrin phosphorylation, the mechanism by which this occurs requires investigation.

3.391 Ultracentrifugation-based approaches to study regulation of Sec6/8 (exocyst) complex function during development of epithelial cell polarity
Yeaman, C.
*Methods **30**, 198-206 (2003)*

The Sec6/8 (exocyst) complex is an essential component of the exocytic apparatus and plays an evolutionarily conserved role in polarized membrane growth. During development of epithelial cell polarity, this cytosolic protein complex is recruited to plasma membrane sites of cell-cell contact, where it facilitates exocytosis to the lateral membrane domain. However, the identity of membrane binding sites for Sec6/8 complex, mechanisms regulating association of Sec6/8 complex with these sites, and the precise function of the complex in polarized trafficking are not known. Biochemical strategies involving differential, rate-zonal, and isopycnic density gradient ultracentrifugation are providing clues to these questions.

3.392 Disruption of the epithelial apical-junction complex by *Helicobacter pylori* CagA
Amieva, M.R., Vogelmann, R., Covacci, A., Tompkins, L.S., Nelson, W.J. and Falkow, S.
*Science, **300**, 1430-1434 (2003)*

Helicobacter pylori translocates the protein CagA into gastric epithelial cells and has been linked to peptic ulcer disease and gastric carcinoma. We show that injected CagA associates with the epithelial tight-junction scaffolding protein ZO-1 and the transmembrane protein junctional adhesion molecule, causing an ectopic assembly of tight-junction components at sites of bacterial attachment, and altering the composition and function of the apical-junctional complex. Long-term CagA delivery to polarized epithelia caused a disruption of the epithelial barrier function and dysplastic alterations in epithelial cell morphology. CagA appears to target *H. pylori* to host cell intercellular junctions and to disrupt junction-mediated functions.

3.393 Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release
Broquet, A.H., Thomas, G., Masliah, J., Trugnan, G. and Bachelet, M.
*J. Biol. Chem., **278**, 21601-21606 (2003)*

Accumulating evidence suggests that some heat shock proteins (Hsps), in particular the 72-kDa inducible Hsp70, associate to the cell membrane and might be secreted through an unknown mechanism to exert important functions in the immune response and signal transduction. We speculated that specialized structures named lipid rafts, known as important platforms for the delivery of proteins to the cell membrane, might be involved in the unknown mechanism ensuring membrane association and secretion of Hsp70. Lipid rafts are sphingolipid-cholesterol-rich structures that have been mainly characterized in polarized epithelial cells and can be isolated as detergent-resistant microdomains (DRMs). Analysis of soluble and DRM fractions prepared from unstressed Caco-2 epithelial cells revealed that Hsp70, and to a lesser extent calnexin, were present in DRM fractions. Increased expression of Hsps, through heat shock or by using drugs acting on protein trafficking or intracellular calcium level, induced an efficient translocation to DRM. We also found that Hsp70 was released by epithelial Caco-2 cells, and this release dramatically increased after heat shock. Drugs known to block the classical secretory pathway were unable to reduce Hsp70 release. By contrast, release of the protein was affected by the raft-disrupting drug methyl-β-cyclodextrin. Our data suggest that lipid rafts are part of a mechanism ensuring the correct functions of Hsps and provide a rational explanation for the observed membrane association and release of Hsp70.

3.394 Organization of the G protein-coupled receptors rhodopsion and opsin in native membranes
Liang, Y., Fotiadis, D., Filipek, S., Saperstein, D.A., Palczewski, K. and Engel, A.
J. Biol. Chem., ***278***, *21655-21662 (2003)*

G protein-coupled receptors (GPCRs), which constitute the largest and structurally best conserved family of signaling molecules, are involved in virtually all physiological processes. Crystal structures are available only for the detergent-solubilized light receptor rhodopsin. In addition, this receptor is the only GPCR for which the presumed higher order oligomeric state in native membranes has been demonstrated (Fotiadis, D., Liang, Y., Filipek, S., Saperstein, D. A., Engel, A., and Palczewski, K (2003) *Nature* 421, 127-128). Here, we have determined by atomic force microscopy the organization of rhodopsin in native membranes obtained from wild-type mouse photoreceptors and opsin isolated from photoreceptors of *Rpe65-/-* mutant mice, which do not produce the chromophore 11-*cis*-retinal. The higher order organization of rhodopsin was present irrespective of the support on which the membranes were adsorbed for imaging. Rhodopsin and opsin form structural dimers that are organized in paracrystalline arrays. The intradimeric contact is likely to involve helices IV and V, whereas contacts mainly between helices I and II and the cytoplasmic loop connecting helices V and VI facilitate the formation of rhodopsin dimer rows. Contacts between rows are on the extracellular side and involve helix I. This is the first semi-empirical model of a higher order structure of a GPCR in native membranes, and it has profound implications for the understanding of how this receptor interacts with partner proteins.

3.395 Novel localization of the DNA-PK complex in lipid rafts: a putative role in the signal transduction pathway of the ionizing radiation response
Lucero, H., Gae, D. and Taccioli, G.E.
J. Biol. Chem., ***278***, *22136-22143 (2003)*

Increased sensitivity to ionizing radiation (IR) has been shown to be due to defects in DNA double-strand break repair machinery. The major pathway in mammalian cells dedicated to the repair of DNA double-strand breaks is by the nonhomologous end-joining machinery. Six components function in this pathway, of which three (Ku70, Ku86, and DNA-PKcs) constitute a protein complex known as DNA-dependent protein kinase (DNAPK). However, it is now recognized that the cellular radiation response is complex, and radiosensitivity may be also regulated at different levels in the radiation signal transduction pathway. In addition to DNA damage, exposure to IR triggers intracellular signaling cascades that overlap with pathways initiated by ligand engagement to a receptor. In this study, we provide evidence for the novel localization of the DNA-PK complex in lipid rafts. We also show this property is not a generalized characteristic of all DNA repair proteins. Furthermore, we have detected Ku86 in yeast lipid rafts. Our results suggest that the components of this complex might be recruited separately to the plasma membrane by tethering with raft-resident proteins. In addition, we found an irradiation-induced differential protein phosphorylation pattern dependent upon DNA-PKcs in lipid rafts. Thus, we speculate that another role for the DNAPKcs subunit and perhaps for the holoenzyme is in the signal transduction of IR response.

3.396 Virus entry, assembly, budding and membrane rafts (Review article)
Chazal, N. and Gerlier, D.
Microbiol. Mol. Biol. Rev., ***67***, *226-237 (2003)*

Specific microdomains of the plasma membrane called rafts appear to be involved in many biological events such as biosynthetic traffic, endocytic traffic, and the signal transduction pathway. Among pathogens, viruses, which are obligate intracellular parasites, are confronted with the plasma membrane during their life cycle. They have to enter their host cells by fusion, permeation, or endocytic vesicle discharge and to exit them by budding or membrane disruption.
In this review, we focus on data supporting the involvement of membrane rafts in the virus replication cycle, their role as a viral entry site, a platform for the assembly of viral components, and a scaffold for the budding of virus from infected cells. The elucidation of these interactions requires a detailed understanding of raft structures and dynamics.

3.397 GAP43 stimulates inositol trisphosphate-mediated calcium release in response to hypotonicity
Caprini, M. et al.
*The EMBO J., **22**, 3004-3014 (2003)*

The identification of osmo/mechanosensory proteins in mammalian sensory neurons is still elusive. We have used an expression cloning approach to screen a human dorsal root ganglion cDNA library to look for proteins that respond to hypotonicity by raising the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We report the unexpected identification of GAP43 (also known as neuromodulin or B50), a membrane-anchored neuronal protein implicated in axonal growth and synaptic plasticity, as an osmosensory protein that augments $[Ca^{2+}]_i$, in response to hypoton-icity. Palmitoylation of GAP43 plays an important role in the protein osmosensitivity. Depletion of intracellular stores or inhibition of phospholipase C (PLC) activity abrogates hypotonicity-evoked, GAP43-mediated $[Ca^{2+}]_i$ elevations. Notably, hypotonicity promoted the selective association of GAP43 with the PLC-δ_1 isoform, and a concomitant Increase in inositol-1,4,5-trisphospbate (IP_3) formation. Collectively, these findings Indicate that hypo-osmotic activation of GAP43 induces Ca^{2+} release from IP_3-sensitive intracellular stores. The osmosensitivity of GAP43 furnishes a mechanistic framework that links axon elongation with phosphoinositide metabolism, spontaneous triggering of cytosolic Ca^{2+} transients and the regulation of actin dynamics and motility at the growth cone in response to temporal and local mechanical forces.

3.398 Presenilin endoproteolysis mediated by an aspartyl protease activity pharmacologically distinct from γ-secretase
Campbell, W.A., Reed, M.L.O., Strahle, J., Wolfe, M.S. and Xia, W.
*J. Neurochem., **85**, 1563-1574 (2003)*

Presenilin (PS)-dependent γ-secretase cleavage is the final proteolytic step in generating amyloid β protein (Aβ), a key peptide involved in the pathogenesis of Alzheimer's disease. PS undergoes endoproteolysis by an unidentified 'presenilinase' to generate the functional N-terminal and C-terminal fragment heterodimers (NTF/CTF) that may harbor the γ-secretase active site. To better understand the relationship between presenilinase and γ-secretase, we characterized the biochemical properties of presenilinase and compared them with those of γ-secretase. Similar to γ-secretase, presenilinase was most active at acidic pH 6.3. Aspartyl protease inhibitor pepstatin A blocked presenilinase activity with an IC_{50} of ~1 μM. Difluoroketone aspartyl protease transition state analogue MW167 was relatively selective for presenilinase (IC_{50} < 1μM) over γ-secretase (IC_{50} - 16 μM). Importantly, removing the transition state mimicking moiety simultaneously abolished both presenilinase and γ-secretase inhibition, suggesting that presenilinase, like γ-secretase, is an aspartyl protease. Interestingly, several of the most potent γ-secretase inhibitors (IC_{50} = 0.3 or 20 μM) failed to block presenilinase activity. Although *de novo* generation of PS1 fragments coincided with production of Aβ *in vitro*, blocking presenilinase activity without reducing pre-existing fragment levels permitted normal *do novo* generation of Aβ and amyloid intracellular domain. Therefore, presenilinase has characteristics of an aspartyl protease, but this activity is distinct from γ-secretase.

3.399 NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals
Delcroix, J-D., Valletta, J.S., Wu, C., Hunt, S.J., Kowal, A.S. and Mobley, W.C.
*Neuron, **39**¸69-84 (2003)*

Target-derived NGF promotes the phenotypic maintenance of mature dorsal root ganglion (DRG) nociceptive neurons. Here, we provide in vivo and in vitro evidence for the presence within DRG neurons of endosomes containing NGF, activated TrkA, and signaling proteins of the Rapl/Erkl/2, p38MAPK, and PI3K/Akt pathways. Signaling endosomes were shown to be retrogradely transported in the isolated sciatic nerve in vitro. NGF injection in the peripheral target of DRG neurons increased the retrograde transport of p-Erkl/2, p-p38, and pAkt in these membranes. Conversely, NGF antibody injections decreased the retrograde transport of p-Erkl/2 and p-p38. Our results are evidence that signaling endosomes, with the characteristics of early endosomes, convey NGF signals from the target of nociceptive neurons to their cell bodies.

3.400 Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins
Drummer, H.E., Maerz, A. and Poumbourios, P.
*FEBS Lett., **546**, 385-390 (2003)*

Hepatitis C virus (HCV) glycoproteins El and E2 are believed to be retained in the endoplasmic reticulum (ER) or *cis*-Golgi compartment via retention signals located in their transmembrane domains. Here we describe the detection of El and E2 at the surface of transiently transfected HEK 293T and Huh7 cells. Surface-localized E1E2 heterodimers presented exclusively as non-covalently associated complexes. Surface-expressed E2 contained *trans*-Golgi modified complex/hybrid type carbohydrate and migrated diffusely between 70 and 90 kDa while intracellular El and E2 existed as high mannose 35 kDa and 70 kDa precursors, respectively. In addition, surface-localized E1E2 heterodimers were incorporated into E1E2-pseudotyped HIV-l particles that were competent for entry into Huh7 cells. These studies suggest that functional HCV glycoproteins are not retained exclusively in the ER and transit through the secretory pathway.

3.401 Distinct endosomal compartments in early trafficking of low density lipoprotein-derived cholesterol
Sugii, S., Reid, P.C., Ohgami, N., Du, H. and Chang, T-Y.
*J. Biol. Chem., **278**, 27180-27189 (2003)*

We previously studied the early trafficking of low density lipoprotein (LDL)-derived cholesterol in mutant Chinese hamster ovary cells defective in Niemann-Pick type Cl (NPC1) using cyclodextrin (CD) to monitor the arrival of cholesterol from the cell interior to the plasma membrane (PM) (Cruz, J. C., Sugii, S., Yu, C., and Chang, T.-Y. *(2000) J. BioL Chem.* 215,4013-4021). We found that newly hydrolyzed cholesterol derived from LDL first appears in certain CD-accessible pool(s), which we assumed to be the PM, before accumulating in the late endosome/lysosome, where NPC1 resides. To determine the identity of the early CD-accessible pool(s), in this study, we performed additional experiments, including the use of revised CD incubation protocols. We found that prolonged incubation with CD (>30 min) caused cholesterol in internal membrane compartment(s) to redistribute to the PM, where it became accessible to CD. In contrast, a short incubation with CD (5-10 min) did not cause such an effect. We also show that one of the early compartments contains acid lipase (AL), the enzyme required for liberating cholesterol from cholesteryl ester in LDL Biochemical and microscopic evidence indicates that most of the AL is present in endocytic compartment(s) distinct from the late endosome/lysosome. Our results suggest that cholesterol is liberated from LDL cholesteryl ester in the hydrolytic compartment containing AL and then moves to the NPC1-containing late endosome/lysosome before reaching the PM or the endoplasmic reticulum.

3.402 Syntaxin 6 regulates Glut4 trafficking in 3T3-L1 adipocytes
Perera, H.K.I., Clarke, M., Morris, N.J., Hong, W., Chamberlain, L.H. and Gould, G.W.
*Mol. Biol. Cell, **14**, 2946-2958 (2003)*

Insulin stimulates the movement of glucose transporter-4 (Glut4)-containing vesicles to the plasma membrane of adipose cells. We investigated the role of post-Golgi t-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) in the trafficking of Glut4 in 3T3-L1 adipocytes. Greater than 85% of syntaxin 6 was found in Glut4-containing vesides, and this t-SNARE exhibited insulin-stimulated movement to the plasma membrane. In contrast, the colocalization of Glut4 with syntaxin 7, 8, or 12/13 was limited and these molecules did not translocate to the plasma membrane. We used adenovirus to overexpress the cytosolic domain of these syntaxins and studied their effects on GIut4 traffic. Overexpression of the cytosolic domain of syntaxin 6 did not affect insulin-stimulated glucose transport, but increased basal deGlc transport and cell surface Glut4 levels. Moreover, the syntaxin 6 cytosolic domain significantly reduced the rate of Glut4 re-internalization after insulin withdrawal and perturbed sub-endosomal Glut4 sorting; the corresponding domains of syntaxins 8 and 12 were without effect. Our data suggest that syntaxin 6 is involved in a membrane-trafficking step that sequesters Glut4 away from traffic destined for the plasma membrane. We speculate that this is at the level of traffic of Glut4 into its unique storage compartment and that syntaxin 16 may be involved.

3.403 Bursts of high-frequency stimulation trigger rapid delivery of pre-existing α-CaMKII mRNA to synapses: a mechanism in dendritic protein synthesis during long-term potentiation in adult awake rats

Havik, B., Rokke, H., Bardsen, K., Davanger, S. and Bramham, C.R.
Eur. J. Neurosci., ***17****, 2679-2689 (2003)*

Messenger ribonucleic acid encoding the alpha-subunit of calcium/calmodulin-dependent protein kinase II *(camkII)* is abundantly and constitutively expressed in dendrites of pyramidal and granule cell neurons of the adult hippocampus. Recent evidence suggests that *camkII* messenger ribonucleic acid is stored in a translationally dormant state within ribonucleic acid storage granules. Delivery of *camkII* messenger ribonucleic acid from sites of storage to sites of translation may therefore be a key step in activity-driven dendritic protein synthesis and synaptic plasticity. Here we explored possible *camkII* trafficking in the context of long-term potentiation in the dentate gyrus of awake, adult rats. Long-term potentiation was induced by patterned high-frequency stimulation, synaptodendrosomes containing pinched-off dendritic spines were obtained from microdissected dentate gyrus, and messenger ribonucleic acid levels were determined by real-time polymerase chain reaction. High-frequency stimulation triggered a rapid 2.5-fold increase in *camkII* messenger ribonucleic acid levels in the synaptodendrosome fraction. This increase occurred in the absence of *camkII* upregulation in the homogenate fraction, indicating trafficking of pre-existing messenger ribonucleic acid to synaptodendrosomes. The elevation in *camkII* messenger ribonucleic acid was paralleled by an increase in protein expression specific to the synaptodendrosome fraction, and followed by depletion of *camkII* message. Activity-dependent regulation of *camkII* messenger ribonucleic acid and protein did not require N-methyl-D-aspartate receptor activation. In contrast, *N*-methyl-D-aspartate receptor activation was required for induction of the immediate early genes *zif268* and *activity-regulated cytoskeleton-associated protein* in dentate gyrus homogenates. The results support a model in which locally stored *camII* messenger ribonucleic acid is rapidly transported to dendritic spines and translated during long-term potentiation in behaving rats.

3.404 Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane

Umebayashi, K. and Nakano, A.
J. Cell Biol., ***161****, 1117-1131 (2003)*

It was known that the uptake of tryptophan is reduced in the yeast *erg6* mutant, which is defective in a late step of ergosterol biosynthesis. Here, we show that this is because the high affinity tryptophan permease Tat2p is not targeted to the plasma membrane. In wild-type cells, the plasma membrane localization of Tat2p is regulated by the external tryptophan concentration. Tat2p is transported from the Golgi apparatus to the vacuole at high tryptophan, and to the plasma membrane at low tryptophan. However, in the *erg6* mutant, Tat2p is missorted to the vacuole at low tryptophan. The plasma membrane targeting of Tat2p is dependent on detergent-insoluble membrane domains, suggesting that sterol affects the sorting through the organization of lipid rafts. The *erg6* mutation also caused missorting to the multivesicular body pathway in late endosomes. Thus, sterol composition is crucial for protein sorting late in the secretory pathway. Tat2p is subject to polyubiquitination, which acts as a vacuolar-targeting signal, and the inhibition of this process suppresses the Tat2p sorting defects of the *erg6* mutant. The sorting mechanisms of Tat2p that depend on both sterol and ubiquitin will be discussed.

3.405 Pharmacological characterization and immunoaffinity purification of metabotropic glutamate receptor from *Drosophila* overexpressed in Sf9 cells

Panneels, V., Eroglu, C., Cronet, P. and Sinning, I.
Prot. Expression Purification, ***20****, 275-282 (2003)*

Metabotropic glutamate receptors (mGluRs) play important roles in the function and regulation of the central nervous system. Structural studies are necessary for the detailed understanding of their mechanisms of action. However, overexpression and purification of functional receptors in quantities required for these studies proves to be a major challenge. In this study we report the overexpression of a *Drosophila melanogaster* mGluR (DmG1uRA) by using a baculovirus—insect cell expression system. Expression was tested in two different insect cell hosts (Sf9 and Hi5) and analyzed by performing expression kinetics. Pharmacological characterization of the recombinant receptor by radioactive glutamate binding assays showed a profile similar to group II mGluRs, as previously reported, when the receptor was expressed in mammalian systems. The B_{max} value reached 11 pmol receptor/mg Sf9-membrane protein. A monoclonal antibody against DmG1uRA was generated by genetic immunization and used to purify the receptor.

3.406 Inhibition of GTP-dependent vesicle trafficking impairs internalization of palsmalemmal eNOS and cellular nitric oxide production

Chaterjee, S., Cao, S., Peterson, T.E., Simari, R.D. and Shah, V.

J. Cell Sci., ***116****, 3645-3655 (2003)*

The Ca^{2+} mobilizing peptide, bradykinin (BK), stimulates endothelial nitric oxide synthase (eNOS)-derived cellular nitric oxide (NO) production in association with altering the subcellular distribution of the enzyme. In the present study we examine the influence of cellular GTPases, particularly the large GTPase dynamin, on BK-mediated eNOS localization and cellular NO production. BK stimulation of ECV cells, which were stably transfected with eNOS-GFP (eNOS-GFP ECV304), increased NO production. This was associated with the mobilization of eNOS-GFP protein into Triton X-100-insoluble fractions of cell lysates, and an internalization of plasmalemmal eNOS-GFP in live and fixed ECV 304 cells. Incubation of digitonin-permeabilized ECV304 cells with the non-hydrolyzed GTP analog, GTPγ-S, abrogated the BK-mediated internalization of eNOS-GFP as assessed by confocal microscopy. Conversely, inhibition of clathrin--dependent endocytosis, via overexpression of AP 180 or pretreatment of cells with chlorpromazine, did not influence BK-mediated eNOS redistribution. Furthermore, specific inhibition of dynamin-2 GTPase function by overexpression of a dominant negative construct, K44A, prevented the BK-mediated enrichment of eNOS-GFP within low buoyant density, caveolin-enriched fractions of eNOS-GFP ECV304 cell lysates. Dynamin-2 K44A overexpression also markedly impaired BK-dependent, L-NAME-inhibited NO production as did incubation of permeabilized cells with GTP-γ-s. These studies demonstrate that disruption of dynamin- and GTPdependent, but clathrin-independent, vesicle trafficking pathways impairs BK-dependent cellular NO production, via inhibition of the internalization of eNOS-containing plasmalemmal vesicles.

3.407 Identification of organelles in bacteria similar to acidocalsisomes of unicellular eukaryotes

Seufferheld, M. et al

J. Biol. Chem., ***278(32)****, 29971-29978 (2003)*

Acidocalcisomes are acidic calcium storage compartments described in several unicellular eukaryotes, including trypanosomatid and apicomplexan parasites, algae, and slime molds. In this work, we report that the volutin granules of *Agrobacterium tumefaciens* possess properties similar to the acidocalcisomes. Transmission electron microscopy revealed that each intracellular granule was surrounded by a membrane. X-ray microanalysis of the volutin granules showed large amounts of phosphorus, magnesium, potassium, and calcium. Calcium in the volutin granules increased when the bacteria were incubated at high extracellular calcium concentration. Immunofluorescence and immunoelectron microscopy, using antisera raised against peptide sequences conserved in the *A. tumefaciens* proton pyrophosphatase, indicated localization in intracellular vacuoles. Purification of the volutin granules using **iodixanol** density gradients indicated a preferential localization of the pyrophosphatase activity in addition to high concentrations of phosphate, pyrophosphate, short- and long-chain polyphosphate, but lack of markers of the plasma membrane. The pyrophosphatase activity was potassium-insensitive and inhibited by the pyrophosphate analogs, amynomethylenediphosphonate and imidodiphosphate, by dicyclohexylcarbodiimide, and by the thiol reagent *N*-ethylmaleimide. Polyphosphate was also localized to the volutin granules by 4',6'-diamino-2-phenylindole staining. The organelles were acidic, as demonstrated by staining with LysoSensor blue DND-167, a dye especially used to detect very acidic compartments in cells, and cycloprodigiosin, a compound isolated from a marine bacterium that has been shown to uncouple proton pyrophosphatase activity acting as a chloride/proton symport. The results suggest that acidocalcisomes arose before the prokaryotic and eukaryotic lineages diverged.

3.408 Association of the Golgi UDP-galactose transporter with UDP-galactose: ceramide galactosyltransferase allows UDP-galactose import in the endoplasmic reticulum

Sprong, H. et al

Mol. Biol. Cell, ***14****, 3482-3493 (2003)*

UDP-galactose reaches the Golgi lumen through the UDP-galactose transporter (UGT) and is used for the galactosylation of proteins and lipids. Ceramides and diglycerides are galactosylated within the endoplasmic reticulum by the UDP-galactose:ceramide galactosyltransferase. It is not known how UDP-galactose is transported from the cytosol into the endoplasmic reticulum. We transfected ceramide galactosyltransferase cDNA into CHOlec8 cells, which have a defective UGT and no endogenous ceramide galactosyltransferase. Cotransfection with the human UGT1 greatly stimulated synthesis of lactosylceramide in the Golgi and of galactosylceramide in the endoplasmic reticulum. UDP-galactose was

directly imported into the endoplasmic reticulum because transfection with UGT significantly enhanced synthesis of galactosylceramide in endoplasmic reticulum membranes. Subcellular fractionation and double label immunofluorescence microscopy showed that a sizeable fraction of ectopically expressed UGT and ceramide galactosyltransferase resided in the endoplasmic reticulum of CHOlec8 cells. The same was observed when UGT was expressed in human intestinal cells that have an endogenous ceramide galactosyltransferase. In contrast, in CHOlec8 singly transfected with UGT 1, the transporter localized exclusively to the Golgi complex. UGT and ceramide galactosyltransferase were entirely detergent soluble and form a complex because they could be coimmunoprecipitated. We conclude that the ceramide galactosyltransferase ensures a supply of UDP-galactose in the endoplasmic reticulum lumen by retaining UGT in a molecular complex.

3.409 Screening for nitric oxide-dependent protein-protein interactions
Matsumoto, A., Comatas, K.E., Liu, L. and Stamler, J.S.
Science, ***301***, *657-661 (2003)*

Because nitric oxide (NO) may be a ubiquitous regulator of cellular signaling, we have modified the yeast two-hybrid system to explore the possibility of NO-dependent protein-protein interactions.We screened for binding partners of procaspase-3, a protein implicated in apoptotic signaling pathways, and identified multiple NO-dependent interactions.Two such interactions, with acid sphingomyelinase and NO synthase, were shown to occur in mammalian cells dependent on endogenous NO.Nitrosylation may thus provide a broad-based mechanism for regulating interactions between proteins.If so, systematic proteomic analyses in which redox state and NO bioavailability are carefully controlled will reveal a large array of novel interactions.

3.410 The yeast deubiquitinating enzyme Ubp16 is anchored to the outer mitochondrial membrane
Kinner, A. and Kölling, R.
FEBS Lett., ***549***, *135-140 (2003)*

We looked for membrane-associated Dubs (deubiquitinating enzymes) among the 16 yeast members of the ubiquitin-specific processing protease (Ubp) family to identify potential regulators of ubiquitin-dependent processes at membranes. For each of the Ubps examined, a certain fraction was found to be membrane associated. This fraction was only small for most Ubps but quite substantial for some Ubps. For Ubp4/Doa4 almost 40% of the protein was found in the membrane fraction suggesting that this protein performs a major function at membranes, probably at endosomes. Among the proteins tested, only one protein (Ubp16) was exclusively membrane associated. By cell fractionation and immunofluorescence experiments, we could show that Ubp16 is localized to mitochondria. Ubp16 contains an N-terminal hydrophobic domain that is similar to N-terminal sequences of other yeast outer mitochondrial membrane proteins. The presence of this putative signal sequence and the result of protease protection experiments suggest that Ubp16 is an integral membrane protein of the outer mitochondrial membrane with an N_{in}–C_{out} orientation. Phenotypic characterization of the Δ*ubp16* mutant and overexpression studies further suggest that Ubp16 is probably not important for the general functioning of mitochondria, but that it rather performs a more specialized function at mitochondria.

3.411 Rotavirus infectious particles use lipid rafts during replication for transport to the cell surface in vitro and in vivo
Cuadras, M.A. and Greeberg, H.B.
Virology, ***313***, *308-321 (2003)*

The pathway by which rotavirus is released from the cell is poorly understood but recent work has shown that, prior to cell lysis, rotavirus is released almost exclusively from the apical surface of the infected cell. By virtue of their unique biochemical and physical properties, viruses have exploited lipid rafts for host cell entry and/or assembly. Here we characterized the association of rhesus rotavirus (RRV) with lipid rafts during the rotavirus replication cycle. We found that newly synthesized infectious virus associates with rafts in vitro and in vivo. RRV proteins cosegregated with rafts on density gradients. Viral infectivity and genomic dsRNA also cosegregated with the raft fractions. Confocal microscopic analysis of raft and RRV virion proteins demonstrated colocalization within the cell. In addition, cholesterol depletion interfered with the association of RRV particles with rafts and reduced the release of infectious particles from the cell. Furthermore, murine rotavirus associates with lipid rafts in intestinal epithelial cells during a natural infection in vivo. Our results confirm the association of rotavirus infectious particles with rafts during

replication in vitro and in vivo and strongly support the conclusion that this virus uses these microdomains for transport to the cell surface during replication.

3.412 Glutamate-binding affinity of *Drosophila* metabotropic glutamate receptor is modulated by association with lipid rafts
Eroglu, C., Brügger, B., Wieland, F. And Sinning, I.
*PNAS, **100(18)**, 10219-10224 (2003)*

Metabotropic glutamate receptors (mGluRs) are responsible for the effects of glutamate in slow synaptic transmission, and are implicated in the regulation of many processes in the CNS. Recently, we have reported the expression and purification of a mGluR from *Drosophila melanogaster* (DmGluRA), a homologue of mammalian group II mGluRs. We have shown that ligand binding to reconstituted DmGluRA requires the presence of ergosterol in the liposomes [Eroglu, C., Cronet, P., Panneels, V., Beaufils, P. & Sinning, I. (2002) *EMBO Rep.* 3, 491-496]. Here we demonstrate that the receptor exists in different affinity states for glutamate, depending on the membrane composition. The receptor is in a high-affinity state when associated with sterol-rich lipid microdomains (rafts), and in a low-affinity state out of rafts. Enrichment of the membranes with cholesterol shifts the receptor into the high-affinity state, and induces its association with rafts. The receptor was crosslinked to photocholesterol. Our data suggest that sterol-rich lipid rafts act as positive allosteric regulators of DmGluRA.

3.413 Mutational analysis of the cytoplasmic domain of β1,4-galactosyltransferase I: influence of phosphorylation on cell surface expression
Hathaway, H.J. et al
*J. Cell Sci., **116**, 4319-4330 (2003)*

β1,4-Galactosyltransferase I (GalT I) exists in two subcellular compartments where it performs two distinct functions. The majority of GalT I is localized in the Golgi complex where it participates in glycoprotein biosynthesis; however, a small portion of GalT I is expressed on the cell surface where it functions as a matrix receptor by binding terminal *N*-acetylglucosamine residues on extracellular glycoside ligands. The GalT I polypeptide occurs in two alternate forms that differ only in the length of their cytoplasmic domains. It is thought that the longer cytoplasmic domain is responsible for GalT I function as a cell surface receptor because of its ability to associate with the detergent-insoluble cytoskeleton. In this study, we demonstrate that the long GalT I cytoplasmic and transmembrane domains are capable of targeting a reporter protein to the plasma membrane, whereas the short cytoplasmic and transmembrane domains do not have this property. The surface-localized GalT I reporter protein partitions with the detergent-insoluble pool, a portion of which co-fractionates with caveolin-containing lipid rafts. Site-directed mutagenesis of the cytoplasmic domain identified a requirement for serine and threonine residues for cell surface expression and function. Replacing either the serine or threonine with aspartic acid reduces surface expression and function, whereas substitution with neutral alanine has no effect on surface expression or function. These results suggest that phosphorylation negatively regulates GalT I function as a surface receptor. Consistent with this, phosphorylation of the endogenous, full-length GalT I inhibits its stable expression on the cell surface. Thus, the 13 amino acid extension unique to the long GalT I isoform is required for GalT I expression on the cell surface, the function of which is regulated by phosphorylation.

3.414 Abnormal cholesterol processing in Alzheimer's disease patient's fibroblasts
Dufour, D., Zhao, W-Q., Ravindranath, L. and Alkon, D.L.
*Neurobiol. Lipids., **1(7)**, 34-44 (2003)*

Cholesterol has recently received attention as a potentially important factor in Alzheimer's disease etiology. Caveolin, which binds cholesterol, plays a prominent role in cellular cholesterol transport. Here, we found a higher level of cholesterol and caveolin in the caveolae-enriched fractions prepared from Alzheimer's disease patients' (AD) fibroblasts compared with age and sex matched controls (AC). Furthermore, the cross-linking activation of the prion protein, which is known to link to signal transduction of caveolin, is altered in AD fibroblasts. Our results suggest a dysregulation of cholesterol processing in AD fibroblasts which may contribute to the pathogenesis of AD.

3.415 Distribution of myocilin, a glaucoma gene product, in human corneal fibroblasts
Wentz-Hunter, K., Shen, X. and Yue, B.Y.J.

*Mol. Vision., **9**, 308-314 (2003)*

Purpose: Myocilin is a gene linked to open-angle glaucomas. In this study, the expression and distribution of myocilin in corneal fibroblasts with or without dexamethasone (DEX) treatment were investigated.
Methods: Human corneal fibroblasts were treated with 100 nM DEX for 10-14 days. Immunofluorescence staining for myocilin was performed. Cell lysates and ultracentrifugation fractions were assessed by western blotting for distribution of myocilin and its possible association with various organelles. Staurosporine was used to induce apoptosis and apoptotic cells were detected using a monoclonal single stranded DNA antibody.
Results: By immunofluorescence, myocilin protein was found to distribute throughout the cytoplasm of corneal fibroblasts including perinuclear regions. Myocilin distribution overlapped to varying degrees with that of the Golgi complex, endoplasmic reticulum, and mitochondria. Subsequent examination by subcellular fractionation however revealed that myocilin, while co-sedimenting with the Golgi complex, lysosomes, and endoplasmic reticulum, did not fractionate or associate with mitochondria. On western blots, protein bands at approximately 66, 57, and 55 kDa were detected and the intensity of the bands was not affected by DEX treatment in corneal fibroblasts. Apoptosis was induced by staurosporine to a similar extent in both DEX-treated and untreated corneal cultures.
Conclusions: In corneal fibroblasts, myocilin expression is not enhanced by DEX treatment and the protein was not associated with mitochondria, in contrast to what were found in human trabecular meshwork (TM) cells. Such differences suggest that the expression and distribution of myocilin may be distinctive for TM cells and may explain why pathology with myocilin mutations is only evident in glaucoma even though myocilin is expressed ubiquitously in ocular and nonocular tissues.

3.416 Proteomic characterisation of neuronal sphingolipid-cholesterol microdomains: role in plasminogen activation
Ledesma, M.D., Da Silva, J.S., Schevchenko, A., Wilm, M. And Dotti, C.G.
*Brain Res., **987**, 107-116 (2003)*

Sorting of certain membrane proteins requires a mechanism involving rafts, protein–lipid complexes enriched in glycosphingolipids and cholesterol. These microdomains remain at the plasma membrane of different cell types and play a role in signal transduction. Although recent reports have begun to describe molecules associated with rafts, their protein composition remains largely unknown, especially in neuronal cells. To address this question, we have purified detergent-insoluble raft fractions (DRMs) from primary cultures of hippocampal neurons. Bidimensional gel analysis and pharmacological raft lipid manipulation allowed the identification of neuronal raft proteins and their characterisation by MALDI-TOF analysis. Enolases were found among the proteins identified and functional studies demonstrate their participation in plasminogen binding. We also show the specific enrichment in rafts of several other plasminogen binding molecules and the exclusive activation of plasminogen to the protease plasmin in these microdomains. These observations suggest that neuronal rafts may play, in addition to intracellular signaling, a role in extracellular/membrane protein proteolysis.

3.417 Characterization of the expression, intracellular localization, and replication complex association of the putative mouse hepatitis virus RNA-dependent RNA polymerase
Brockway, S.M., Clay, C.T., Lu, X.T. and Denison, M.R.
*J. Virol., **77(19)**, 10515-10527 (2003)*

Mouse hepatitis virus (MHV) RNA synthesis is mediated by a viral RNA-dependent RNA polymerase (RdRp) on membrane-bound replication complexes in the host cell cytoplasm. However, it is not known how the putative MHV RdRp (Pol) is targeted to and retained on cellular membranes. In this report, we show that a 100-kDa protein was stably detected by an anti-Pol antiserum as a mature product throughout the virus life cycle. Gradient fractionation and biochemical extraction experiments demonstrated that Pol was not an integral membrane protein but was tightly associated with membranes and coimmunoprecipitated with the replicase proteins 3CLpro, p22, and p12. By immunofluorescence confocal microscopy, Pol colocalized with viral proteins at replication complexes, distinct from sites of virion assembly, over the entire course of infection. To determine if Pol associated with cellular membranes in the absence of other viral factors, the *pol* domain of gene 1 was cloned and expressed in cells as a fusion with green fluorescent protein, termed Gpol. In Gpol-expressing cells that were infected with MHV, but not in mock-infected cells, Gpol relocalized from a diffuse distribution in the cytoplasm to punctate foci that colocalized with markers for replication complexes. Expression of Gpol deletion mutants established that the conserved enzymatic domains of Pol were dispensable for replication complex association, but a 38-

amino-acid domain in the RdRp unique region of Pol was required. This study demonstrates that viral or virus-induced factors are necessary for Pol to associate with membranes of replication complexes, and it identifies a defined region of Pol that may mediate its interactions with those factors.

3.418 Endoplasmic reticulum export of glycosyltransferases depends on interaction of a cytoplasmic dibasic motif with Sar1
Giraudo, C.G. and Maccioni, J.F.
*Mol. Biol. Cell, **14,** 3753-3766 (2003)*

Membrane proteins exit the endoplasmic reticulum (ER) in COPII-transport vesicles. ER export is a selective process in which transport signals present in the cytoplasmic tail (CT) of cargo membrane proteins must be recognized by coatomer proteins for incorporation in COPII vesicles. Two classes of ER export signals have been described for type I membrane proteins, the diacidic and the dihydrophobic motifs. Both motifs participate in the Sar1-dependent binding of Sec23p–Sec24p complex to the CTs during early steps of cargo selection. However, information concerning the amino acids in the CTs that interact with Sar1 is lacking. Herein, we describe a third class of ER export motif, [RK](X)[RK], at the CT of Golgi resident glycosyltransferases that is required for these type II membrane proteins to exit the ER. The dibasic motif is located proximal to the transmembrane border, and experiments of cross-linking in microsomal membranes and of binding to immobilized peptides showed that it directly interacts with the COPII component Sar1. Sar1GTP-bound to immobilized peptides binds Sec23p. Collectively, the present data suggest that interaction of the dibasic motif with Sar1 participates in early steps of selection of Golgi resident glycosyltransferases for transport in COPII vesicles.

3.419 Endostatin associates with lipid rafts and induces reorganisation of the actin cytoskeleton via down-regulation of RhoA activity
Wickström, S.A., Alitalo, K. and Keski-Oja, J.
*J. Biol. Chem., **278(39)**, 37895-37901 (2003)*

Endostatin, the C-terminal fragment of collagen XVIII, is a potent inhibitor of angiogenesis. Observations that endostatin inhibits endothelial cell migration and induces disassembly of the actin cytoskeleton provide putative cellular mechanisms for this effect. To understand the mechanisms of endostatin-induced intracellular signaling, we analyzed the association of recombinant endostatin with endothelial cell lipid rafts and the roles of its heparin- and integrin-binding properties in this interaction. We observed that a fraction of cell surface-bound endostatin partitioned in low density membrane raft fractions together with caveolin-1. Heparinase treatment of cells prevented the recruitment of endostatin to the lipid rafts but did not affect the association of endostatin with the non-raft fraction, whereas preincubation of endostatin with soluble $\alpha_5\beta_1$ integrin prevented the association of endostatin with the endothelial cell membrane. Endostatin treatment induced recruitment of $\alpha_5\beta_1$ integrin into the raft fraction via a heparan sulfate proteoglycan-dependent mechanism. Subsequently, through $\alpha_5\beta_1$ integrin, heparan sulfate, and lipid raft-mediated interactions, endostatin induced Src-dependent activation of p190RhoGAP with concomitant decrease in RhoA activity and disassembly of actin stress fibers and focal adhesions. These observations provide a cell biological mechanism, which plausibly explains the anti-angiogenic mechanisms of endostatin *in vivo*.

3.420 Phagosomes are competent organelles for antigen cross-presentation
Houde, M. et al
*Nature, **425**, 402-406 (2003)*

The ability to process microbial antigens and present them at the surface of cells is an important aspect of our innate ability to clear infections. It is generally accepted that antigens in the cytoplasm are loaded in the endoplasmic reticulum and presented at the cell surface on major histocompatibility complex (MHC) class I molecules, whereas peptides present in endo/phagocytic compartments are presented on MHC class II molecules. Despite the apparent segregation of the class I and class II pathways, antigens from intracellular pathogens including mycobacteria, *Escherichia coli*, *Salmonella typhimurium*, *Brucella abortus* and *Leishmania*, have been shown to elicit an MHC class-I-dependent $CD8^+$ T-cell response, a process referred to as cross-presentation. The cellular mechanisms allowing the cross-presentation pathway are poorly understood. Here we show that phagosomes display the elements and properties needed to be self-sufficient for the cross-presentation of exogenous antigens, a newly ascribed function linked to phagocytosis mediated by the endoplasmic reticulum.

3.421 Compartmentalization of integrin α6β4 signaling in lipid rafts
Gagnoux-Palacios, L. et al
*J. Cell Biol., **162(7)**, 1189-1196 (2003)*

Integrin α6ß4 signaling proceeds through Src family kinase (SFK)–mediated phosphorylation of the cytoplasmic tail of ß4, recruitment of Shc, and activation of Ras and phosphoinositide-3 kinase. Upon cessation of signaling, α6ß4 mediates assembly of hemidesmosomes. Here, we report that part of α6ß4 is incorporated in lipid rafts. Metabolic labeling in combination with mutagenesis indicates that one or more cysteine in the membrane-proximal segment of ß4 tail is palmitoylated. Mutation of these cysteines suppresses incorporation of α6ß4 in lipid rafts, but does not affect α6ß4-mediated adhesion or assembly of hemidesmosomes. The fraction of α6ß4 localized to rafts associates with a palmitoylated SFK, whereas the remainder does not. Ligation of palmitoylation-defective α6ß4 does not activate SFK signaling to extracellular signal–regulated kinase and fails to promote keratinocyte proliferation in response to EGF. Thus, compartmentalization in lipid rafts is necessary to couple the α6ß4 integrin to a palmitoylated SFK and promote EGF-dependent mitogenesis.

3.422 Mislocalization of membrane proteins associated with multidrug resistance in cisplastin-resistant cancer cell lines
Liang, X-J., Shen, D-W., Garfield, S. And Gottesman, M.M.
*Cancer Res., **63**, 5909-5916 (2003)*

The accumulation of [^{14}C]carboplatin and [^{3}H]methotrexate is reduced in single-step KB epidermoid adenocarcinoma (KB-CP) cells, which are cross-resistant to carboplatin, methotrexate, and sodium arsenite. In these KB-CP cells, multidrug resistance is accompanied by mislocalization of multidrug resistance associated protein (MRP) 1 and other membrane proteins such as folate-binding protein. MRP1 was not decreased in amount in single-step variants but accumulates in a cytoplasmic fraction, and its apparent molecular weight was altered probably because of reduced glycosylation in resistant cells. This low-density compartment was partially labeled with antibodies to lectin-GSII (a Golgi marker) and Bip/GRP78 (an endoplasmic reticulum marker). Pulse-chase labeling of MRP1 with ^{35}S-methionine and ^{35}S-cysteine and pulse-chase biotinylation of cell surface MRP1 suggests that membrane protein mislocalization is caused mainly by a defect of plasma membrane protein recycling, manifested also as a defect in acidification of lysosomes. The reduced accumulation of cytotoxic compounds in the KB-CP cells is presumed to result from the failure of carrier proteins and/or transporters to localize to the plasma membrane.

3.423 The major surface protease (MSP or GP63) of *Leishmania* sp. Biosynthesis, regulation of expression and function
Yao, C., Donelson, J.E. and Wilson, M.E.
*Mol. Biol. Parasitol., **132**, 1-16 (2003)*

Leishmania sp. are digenetic protozoa that cause an estimated 1.5–2 million new cases of leishmaniasis per year worldwide. Among the molecular factors that contribute to *Leishmania* sp. virulence and pathogenesis is the major surface protease, alternately called MSP, GP63, leishmanolysin, EC3.4.24.36, and PSP, which is the most abundant surface protein of leishmania promastigotes. Recent studies using gene knockout, antisense RNA and overexpression mutants have demonstrated a role for MSP in resistance of promastigotes to complement-mediated lysis and either a direct or indirect role in receptor-mediated uptake of leishmania. The *MSP* gene clusters in different *Leishmania* sp. include multiple distinct *MSP*s that tend to fall into three classes, which can be distinguished by their sequences and by their differential expression in parasite life stages. Regulated expression of *MSP* class gene products during the parasite life cycle occurs at several levels involving both mRNA and protein metabolism. In this review we summarize advances in MSP research over the past decade, including organization of the gene families, crystal structure of the protein, regulation of mRNA and protein expression, biosynthesis and possible functions. The MSPs exquisitely demonstrate the multiple levels of post-transcriptional gene regulation that occur in *Leishmania* sp. and other trypanosomatid protozoa.

3.424 APP processing is regulated by cytoplasmic phosphorylation
Lee, M-S. et al
*J. Cell Biol., **163(1)**, 83-95 (2003)*

Amyloid-ß peptide (Aß) aggregate in senile plaque is a key characteristic of Alzheimer's disease (AD). Here, we show that phosphorylation of amyloid precursor protein (APP) on threonine 668 (P-APP) may play a role in APP metabolism. In AD brains, P-APP accumulates in large vesicular structures in afflicted hippocampal pyramidal neurons that costain with antibodies against endosome markers and the ß-secretase, BACE1. Western blot analysis reveals increased levels of T668-phosphorylated APP COOH-terminal fragments in hippocampal lysates from many AD but not control subjects. Importantly, P-APP cofractionates with endosome markers and BACE1 in an iodixanol gradient and displays extensive colocalization with BACE1 in rat primary cortical neurons. Furthermore, APP COOH-terminal fragments generated by BACE1 are preferentially phosphorylated on T668 verses those produced by α-secretase. The production of Aß is significantly reduced when phosphorylation of T668 is either abolished by mutation or inhibited by T668 kinase inhibitors. Together, these results suggest that T668 phosphorylation may facilitate the BACE1 cleavage of APP to increase Aß generation.

3.425 **Functional γ-secretase complex assembly in Golgi/*trans*-Golgi network: interactions among presenilin, nicastrin, Aph1, Pen-2, and γ-secreatase substrates**
Baulac, S. et al
Neurobiology of Disease, ***14***, *194-204 (2003)*

γ-Secretase is a proteolytic complex whose substrates include Notch, β-amyloid precursor protein (APP), and several other type I transmembrane proteins. Presenilin (PS) and nicastrin are known components of this high-molecular-weight complex, and recent genetic screens in invertebrates have identified two additional gene products, Aph1 and Pen-2, as key factors in γ-secretase activity. Here, we examined the interaction of the components of the γ-secretase complex in Chinese hamster ovary cells stably expressing human forms of APP, PS1, Aph1, and Pen-2. Subcellular fractionation of membrane vesicles and subsequent coimmunoprecipitation of individual γ-secretase components revealed that interactions among all proteins occurred in the Golgi/*trans*-Golgi network (TGN) compartments. Furthermore, incubation of the Golgi/TGN-enriched vesicles resulted in de novo generation of amyloid β-protein and APP intracellular domain. Immunofluorescent staining of the individual γ-secretase components supported our biochemical evidence that the γ-secretase components assemble into the proteolytically active γ-secretase complex in the Golgi/TGN compartment.

3.426 **The conversion of apoB100 low density lipoprotein/high density lipoprotein particles to apoB100 very low density lipoproteins in response to oleic acid occurs in the endoplasmic reticulum and not in the Golgi in Mca RH7777 cells**
Yamaguchi, J., Gamble, M.V., Conlon, D., Liang, J-S. and Ginsberg, H.N.
J. Biol. Chem., ***278(43)***, *42643-42651 (2003)*

The site where bulk lipid is added to apoB100 low density lipoproteins (LDL)/high density lipoproteins (HDL) particles to form triglyceride-enriched very low density lipoproteins (VLDL) has not been identified definitively. We employed several strategies to address this question. First, McA RH7777 cells were pulse-labeled for 20 min with [^{35}S]methionine/cysteine and chased for 1 h (Chase I) to allow study of newly synthesized apoB100 LDL/HDL remaining in the endoplasmic reticulum (ER). After Chase I, cells were incubated for another hour (C2) with/without brefeldin A (BFA) and nocodazole (Noc) (to block ER to Golgi trafficking) and with/without oleic acid (OA). OA treatment alone during C2 increased VLDL secretion. This was prevented by the addition of BFA/Noc in C2. When C2 media were replaced by control media for another 1-h chase (C3), VLDL formed during OA treatment in C2 were secreted into C3 medium. Thus, OA-induced conversion of apoB100 LDL/HDL to VLDL during C2 occurred in the ER. Next, newly synthesized apoB100 lipoproteins were trapped in the Golgi by treatment with Noc and monensin during Chase I (C1), and C2 was carried out in the presence of BFA/Noc with/without OA and without monensin. Under these conditions, OA treatment during C2 did not stimulate VLDL secretion. The same pulse/chase protocols were followed by **iodixanol** subcellular fractionation, extraction of lipoproteins from ER and Golgi, and sucrose gradient separation of extracted lipoproteins. Cells treated with BFA/Noc and OA in C2 had VLDL in the ER. In the absence of OA, only LDL/HDL were present in the ER. The density of Golgi lipoproteins in these cells was not affected by OA. Similar results were obtained when ER were immuno-isolated with anti-calnexin antibodies. In conclusion, apoB100 bulk lipidation, resulting in conversion of LDL/HDL to VLDL, can occur in the ER, but not in the Golgi, in McA RH7777 cells.

3.427 **Pressure-induced differential regulation of the two tryptophan permeases Tat1 and Tat2 by ubiquitin ligase Rsp5 and its binding proteins, Bu11 and Bu12**
Abe, F. and Iida, H.

Mol. Cell. Biol., ***23(21)***, *7566-7584 (2003)*

Tryptophan uptake appears to be the Achilles' heel in yeast physiology, since under a variety of seemingly diverse toxic conditions, it becomes the limiting factor for cell growth. When growing cells of *Saccharomyces cerevisiae* are subjected to high hydrostatic pressure, tryptophan uptake is down-regulated, leading to cell cycle arrest in the G_1 phase. Here we present evidence that the two tryptophan permeases Tat1 and Tat2 are differentially regulated by Rsp5 ubiquitin ligase in response to high hydrostatic pressure. Analysis of high-pressure growth mutants revealed that the *HPG1* gene was allelic to *RSP5*. The *HPG1* mutation or the *bul1*Δ *bul2*Δ double mutation caused a marked increase in the steady-state level of Tat2 but not of Tat1, although both permeases were degraded at high pressure in an Rsp5-dependent manner. There were marked differences in subcellular localization. Tat1 localized predominantly in the plasma membrane, whereas Tat2 was abundant in the internal membranes. Moreover, Tat1 was associated with lipid rafts, whereas Tat2 localized in bulk lipids. Surprisingly, Tat2 became associated with lipid rafts upon the occurrence of a ubiquitination defect. These results suggest that ubiquitination is an important determinant of the localization and regulation of these tryptophan permeases. Determination of the activation volume ($\Delta V^{\neq}$) for Tat1- and Tat2-mediated tryptophan uptake (89.3 and 50.8 ml/mol, respectively) revealed that both permeases are highly sensitive to membrane perturbation and that Tat1 rather than Tat2 is likely to undergo a dramatic conformational change during tryptophan import. We suggest that hydrostatic pressure is a unique tool for elucidating the dynamics of integral membrane protein functions as well as for probing lipid microenvironments where they localize.

3.428 The AP-1A and AP1B clathrin adaptor complexes define biochemically and functionally distinct membrane domains

Fölsch, H., Pypaert, M., Maday, S., Pelletier, L. and Mellman, I.
J. Cell Biol., ***163(2)***, *351-362 (2003)*

Most epithelial cells contain two AP-1 clathrin adaptor complexes. AP-1A is ubiquitously expressed and involved in transport between the TGN and endosomes. AP-1B is expressed only in epithelia and mediates the polarized targeting of membrane proteins to the basolateral surface. Both AP-1 complexes are heterotetramers and differ only in their 50-kD μ1A or μ1B subunits. Here, we show that AP-1A and AP-1B, together with their respective cargoes, define physically and functionally distinct membrane domains in the perinuclear region. Expression of AP-1B (but not AP-1A) enhanced the recruitment of at least two subunits of the exocyst complex (Sec8 and Exo70) required for basolateral transport. By immunofluorescence and cell fractionation, the exocyst subunits were found to selectively associate with AP-1B–containing membranes that were both distinct from AP-1A–positive TGN elements and more closely apposed to transferrin receptor–positive recycling endosomes. Thus, despite the similarity of the two AP-1 complexes, AP-1A and AP-1B exhibit great specificity for endosomal transport versus cell polarity.

3.429 Epstein-Barr virus latent membrane protein 2A activates β-catenin signaling in epithelial cells

Morrison, J.A., Klingelhutz, A.J. and Raab-Traub, N.
J. Virol., ***77(22)***, *12276-12284 (2003)*

The Epstein-Barr virus (EBV) latent membrane protein 2A (LMP2A) functions to maintain latency in EBV-infected B lymphocytes. Although LMP2A is nonessential for the immortalization of B lymphocytes by EBV, its expression in B lymphocytes prevents viral reactivation by blocking B-cell receptor activation and signaling. LMP2A also provides an antiapoptotic signal in transgenic mice that express LMP2A in B lymphocytes. LMP2A activates phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase Akt in lymphocytes and epithelial cells. Here we show that EBV LMP2A activates the PI3K and ß-catenin signaling pathways in telomerase-immortalized human foreskin keratinocytes (HFK). LMP2A activated Akt in a PI3K-dependent manner, and the downstream Akt targets glycogen synthase kinase 3ß (GSK3ß) and the Forkhead transcription factor FKHR were phosphorylated and inactivated in LMP2A-expressing HFK cells. GSK3ß is a negative regulator of the Wnt signaling pathway, and inactivation of GSK3ß by LMP2A signaling led to stabilization of ß-catenin, the central oncoprotein of Wnt signaling. In LMP2A-expressing cells, ß-catenin accumulated in the cytoplasm and translocated into the nucleus via a two-step mechanism. The cytoplasmic accumulation of ß-catenin downstream of LMP2A was independent of PI3K signaling, whereas its nuclear translocation was dependent on PI3K signaling. In the nucleus, ß-catenin activated a reporter responsive to T-cell factor, and this activation was augmented by LMP2A coexpression. The Wnt pathway is inappropriately activated in 90% of colon cancers and is dysregulated in several other cancers, and these data suggest that activation of this pathway by LMP2A may contribute to the generation of EBV-associated cancers.

3.430 Function, expression and localization of annexin A7 in platelets and red blood cells: insights derived from an annexin A7 mutant mouse
Herr, C. et al
BMC Biochem., ***4(8)****, 1-11 (2003)*

Annexin A7 is a Ca^{2+}- and phospholipid-binding protein expressed as a 47 and 51 kDa isoform, which is thought to be involved in membrane fusion processes. Recently the 47 kDa isoform has been identified in erythrocytes where it was proposed to be a key component in the process of the Ca^{2+}-dependent vesicle release, a process with which red blood cells might protect themselves against an attack by for example complement components.
The role of annexin A7 in red blood cells was addressed in erythrocytes from *anxA7*$^{-/-}$ mice. Interestingly, the Ca^{2+}-mediated vesiculation process was not impaired. Also, the membrane organization appeared not to be disturbed as assessed using gradient fractionation studies. Instead, lack of annexin A7 led to an altered cell shape and increased osmotic resistance of red blood cells. Annexin A7 was also identified in platelets. In these cells its loss led to a slightly slower aggregation velocity which seems to be compensated by an increased number of platelets. The results appear to rule out an important role of annexin A7 in membrane fusion processes occurring in red blood cells. Instead the protein might be involved in the organization of the membrane cytoskeleton. Red blood cells may represent an appropriate model to study the role of annexin A7 in cellular processes.

We have demonstrated the presence of both annexin A7 isoforms in red blood cells and the presence of the small isoform in platelets. In both cell types the loss of annexin A7 impairs cellular functions. The defects observed are however not compatible with a crucial role for annexin A7 in membrane fusion processes in these cell types.

3.431 Vesicular localization and activity-dependent trafficking of presynaptic choline transporters
Ferguson, S.M. et al
J. Neurosci., ***23(30)****, 9697-9709 (2003)*

Presynaptic synthesis of acetylcholine (ACh) requires a steady supply of choline, acquired by a plasma membrane, hemicholinium-3-sensitive (HC-3) choline transporter (CHT). A significant fraction of synaptic choline is recovered from ACh hydrolyzed by acetylcholinesterase (AChE) after vesicular release. Although antecedent neuronal activity is known to dictate presynaptic CHT activity, the mechanisms supporting this regulation are unknown. We observe an exclusive localization of CHT to cholinergic neurons and demonstrate that the majority of CHTs reside on small vesicles within cholinergic presynaptic terminals in the rat and mouse brain. Furthermore, immunoisolation of presynaptic vesicles with multiple antibodies reveals that CHT-positive vesicles carry the vesicular acetylcholine transporter (VAChT) and synaptic vesicle markers such as synaptophysin and Rab3A and also contain acetylcholine. Depolarization of synaptosomes evokes a Ca^{2+}-dependent botulinum neurotoxin C-sensitive increase in the V_{max} for HC-3-sensitive choline uptake that is accompanied by an increase in the density of CHTs in the synaptic plasma membrane. Our study leads to the novel hypothesis that CHTs reside on a subpopulation of synaptic vesicles in cholinergic terminals that can transit to the plasma membrane in response to neuronal activity to couple levels of choline re-uptake to the rate of ACh release.

3.432 Visualization of protein compartmentation within the plasma membrane of living yeast cells
Malinska, K., Malinsky, J., Opekarova, M and Tanner, W.
Mol. Biol. Cell., ***14(11)****, 4427-4436 (2003)*

Different distribution patterns of the arginine/H^+ symporter Can1p, the H^+ plasma membrane ATPase Pma1p, and the hexose transport facilitator Hxt1p within the plasma membrane of living *Saccharomyces cerevisiae* cells were visualized using fluorescence protein tagging of these proteins. Although Hxt1p-GFP was evenly distributed through the whole cell surface, Can1p-GFP and Pma1p-GFP were confined to characteristic subregions in the plasma membrane. Pma1p is a well-documented raft protein. Evidence is presented that Can1p, but not Hxt1p, is exclusively associated with lipid rafts, too. Double labeling experiments with Can1p-GFP– and Pma1p-RFP–containing cells demonstrate that these proteins occupy two different nonoverlapping membrane microdomains. The size of Can1p-rich (Pma1p-poor) areas was estimated to 300 nm. These domains were shown to be stable in growing cells for >30 min. To our knowledge, this is the first observation of a cell polarization-independent lateral compartmentation in the plasma membrane of a living cell.

3.433 Inhibition of heme biosynthesis prevents transcription of iron uptake genes in yeast
Crisp, R.J. et al
J. Biol. Chem., ***278(46)***, *45499-45506 (2003)*

Yeast are capable of modifying their metabolism in response to environmental changes. We investigated the activity of the oxygen-dependent high-affinity iron uptake system of *Saccharomyces cerevisiae* under conditions of heme depletion. We found that the absence of heme, due to a deletion in the gene that encodes δ-aminolevulinic acid synthase (*HEM1*), resulted in decreased transcription of genes belonging to both the iron and copper regulons, but not the zinc regulon. Decreased transcription of the iron regulon was not due to decreased expression of the iron sensitive transcriptional activator Aft1p. Expression of the constitutively active allele *AFT1-1*^{up} was unable to induce transcription of the high affinity iron uptake system in heme-depleted cells. We demonstrated that under heme-depleted conditions, Aft1p-GFP was able to cycle normally between the nucleus and cytosol in response to cytosolic iron. Despite the inability to induce transcription under low iron conditions, chromatin immunoprecipitation demonstrated that Aft1p binds to the *FET3* promoter in the absence of heme. Finally, we provide evidence that under heme-depleted conditions, yeast are able to regulate mitochondrial iron uptake and do not accumulate pathologic iron concentrations, as is seen when iron-sulfur cluster synthesis is disrupted.

3.434 Syndecan-1 transmembrane and extracellular domains have unique and distinct roles in cell spreading
McQuade, K.J. and Repraeger, A.C.
J. Biol. Chem., ***278(47)***, *46607-46615 (2003)*

Raji cells expressing syndecan-1 (Raji-S1) adhere and spread when plated on heparan sulfate-binding extracellular matrix ligands or monoclonal antibody 281.2, an antibody directed against the syndecan-1 extracellular domain. Cells plated on monoclonal antibody 281.2 initially extend a broad lamellipodium, a response accompanied by membrane ruffling at the cell margin. Membrane ruffling then becomes polarized, leading to an elongated cell morphology. Previous work demonstrated that the syndecan-1 cytoplasmic domain is not required for these activities, suggesting important roles for the syndecan-1 transmembrane and/or extracellular domains in the assembly of a signaling complex necessary for spreading. Work described here demonstrates that truncation of the syndecan-1 extracellular domain does not affect the initial lamellipodial extension in the Raji-S1 cells but does inhibit the active membrane ruffling that is necessary for cell polarization. Replacement of the entire syndecan-1 transmembrane domain with leucine residues completely blocks the cell spreading. These data demonstrate that the syndecan-1 transmembrane and extracellular domains have important but distinct roles in Raji-S1 cell spreading; the extracellular domain mediates an interaction that is necessary for dynamic cytoskeletal rearrangements whereas an interaction of the transmembrane domain is required for the initial spreading response.

3.435 Raft disorganization leads to reduced plasmin activity in Alzheimer's disease brains
Ledesma, M.D. et al
EMBO Reports, ***4(12)***, *1190-1196 (2003)*

The serine protease plasmin can efficiently degrade amyloid peptide in vitro, and is found at low levels in the hippocampus of patients with Alzheimer's disease (AD). The cause of such paucity remains unknown. We show here that the levels of total brain plasminogen and plasminogen-binding molecules are normal in these brain samples, yet plasminogen membrane binding is greatly reduced. Biochemical analysis reveals that the membranes of these brains have a mild, still significant, cholesterol reduction compared to age-matched controls, and anomalous raft microdomains. This was reflected by the loss of raft-enriched proteins, including plasminogen-binding and -activating molecules. Using hippocampal neurons in culture, we demonstrate that removal of a similar amount of membrane cholesterol is sufficient to induce raft disorganization, leading to reduced plasminogen membrane binding and low plasmin activity. These results suggest that brain raft alterations may contribute to AD by rendering the plasminogen system inefficient.

3.436 Novel cadherin-related membrane proteins, alcadeins, enhance the X11-like protein-mediated stabilization of amyloid β-protein precursor metabolism
Araki, Y. et al
J. Biol.Chem., ***278(49)***, *49448-49458 (2003)*

Previously we found that X11-like protein (X11L) associates with amyloid β-protein precursor (APP). X11L stabilizes APP metabolism and suppresses the secretion of the amyloid β-protein (Aβ) that are the pathogenic agents of Alzheimer's disease (AD). Here we found that Alcadein (Alc), a novel membrane protein family that contains cadherin motifs and originally reported as calsyntenins, also interacted with X11L. Alc was abundant in the brain and occurred in the same areas of the brain as X11L. X11L could simultaneously associate with APP and Alc, resulting in the formation of a tripartite complex in brain. The tripartite complex stabilized intracellular APP metabolism and enhanced the X11L-mediated suppression of Aβ secretion that is due to the retardation of intracellular APP maturation. X11L and Alc also formed another complex with C99, a carboxyl-terminal fragment of APP cleaved at the β-site (CTFβ). The formation of the Alc·X11L·C99 complex inhibited the interaction of C99 with presenilin, which strongly suppressed the γ-cleavage of C99. In AD patient brains, Alc and APP were particularly colocalized in dystrophic neurites in senile plaques. Deficiencies in the X11L-mediated interaction between Alc and APP and/or CTFβ enhanced the production of Aβ, which may be related to the development or progression of AD.

3.437 RGS16 inhibits signaling through the Gα13-Rho
Johnson, E.N. et al
*Nature Cell Biol., **5(12)**, 1095-1103 (2003)*

Gα13 stimulates the guanine nucleotide exchange factors (GEFs) for Rho, such as p115Rho-GEF[1]. Activated Rho induces numerous cellular responses, including actin polymerization, serum response element (SRE)-dependent gene transcription and transformation[2]. p115Rho-GEF contains a Regulator of G protein Signalling domain (RGS box) that confers GTPase activating protein (GAP) activity towards Gα12 and Gα13 (ref. 3). In contrast, classical RGS proteins (such as RGS16 and RGS4) exhibit RGS domain-dependent GAP activity on Gαi and Gαq, but not Gα12 or Gα13 (ref 4). Here, we show that RGS16 inhibits Gα13-mediated, RhoA-dependent reversal of stellation and SRE activation. The RGS16 amino terminus binds Gα13 directly, resulting in translocation of Gα13 to detergent-resistant membranes (DRMs) and reduced p115Rho-GEF binding. RGS4 does not bind Gα13 or attenuate Gα13-dependent responses, and neither RGS16 nor RGS4 affects Gα12-mediated signalling. These results elucidate a new mechanism whereby a classical RGS protein regulates Gα13-mediated signal transduction independently of the RGS box.

3.438 The lack of annexin A7 affects functions of primary astrocytes
Clemen, C.S., Herr, C., Hövelmeyer, N. and Noegel, A.A.
*Exp. Cell Res., **291**, 406-414 (2003)*

Annexin A7 is a Ca^{2+}- and phospholipid-binding protein, which is thought to function in membrane organization and Ca^{2+}-dependent signaling processes. It localizes to different cellular compartments and exists in a 47- and 51-kDa isoform with the large isoform being expressed in brain, skeletal, and heart muscle. In human temporal brain annexin A7 was found exclusively in astroglial cells. As astrocytes are thought to play key roles in several processes of the brain we focused on Ca^{2+}-dependent signaling processes and astrocyte proliferation. Primary astrocytes from an *anxA7*$^{-/-}$ mouse exhibited an increased velocity of mechanically induced astrocytic Ca^{2+} waves as compared to wild type. We also observed a remarkably increased proliferation rate in cultured mutant astrocytes. A search for annexin A7 binding partners with advanced biochemical methods confirmed sorcin as the major binding protein. However, in vivo GFP-tagged annexin A7 and sorcin appeared to redistribute mainly independently from each other in wild type and in mutant astrocytes. Our results favor an involvement of annexin A7 in Ca^{2+}-dependent signaling or Ca^{2+} homeostasis in astrocytes.

3.439 Subcellular localization and regulation of coenzyme A synthetase
Zhyvoloup, A. et al
*J. Biol. Chem., **278(50)**, 50316-50321 (2003)*

CoA synthase mediates the last two steps in the sequence of enzymatic reactions, leading to CoA biosynthesis. We have recently identified cDNA for CoA synthase and demonstrated that it encodes a bifunctional enzyme possessing 4'-phosphopantetheine adenylyltransferase and dephospho-CoA kinase activities. Molecular cloning of CoA synthase provided us with necessary tools to study subcellular localization and the regulation of this bifunctional enzyme. Transient expression studies and confocal microscopy allowed us to demonstrate that full-length CoA synthase is associated with the mitochondria,

whereas the removal of the N-terminal region relocates the enzyme to the cytosol. In addition, we showed that the N-terminal sequence of CoA synthase (amino acids 1–29) exhibits a hydrophobic profile and targets green fluorescent protein exclusively to mitochondria. Further analysis, involving subcellular fractionation and limited proteolysis, indicated that CoA synthase is localized on the mitochondrial outer membrane. Moreover, we demonstrate for the first time that phosphatidylcholine and phosphatidylethanolamine, which are the main components of the mitochondrial outer membrane, are potent activators of both enzymatic activities of CoA synthase *in vitro*. Taken together, these data provide the evidence that the final stages of CoA biosynthesis take place on mitochondria and the activity of CoA synthase is regulated by phospholipids.

3.440 Yeast homotypic vacuole fusion requires the Ccz1-Mon1 complex during the tethering/docking stage
Wang, C-W., Stromhaug, P.E., Kauffman, E.J., Weisman, L.S. and Klionsky, D.J.
J. Cell Biol., ***163(5)***, *973-985 (2003)*

The function of the yeast lysosome/vacuole is critically linked with the morphology of the organelle. Accordingly, highly regulated processes control vacuolar fission and fusion events. Analysis of homotypic vacuole fusion demonstrated that vacuoles from strains defective in the *CCZ1* and *MON1* genes could not fuse. Morphological evidence suggested that these mutant vacuoles could not proceed to the tethering/docking stage. Ccz1 and Mon1 form a stable protein complex that binds the vacuole membrane. In the absence of the Ccz1–Mon1 complex, the integrity of vacuole SNARE pairing and the unpaired SNARE class C Vps/HOPS complex interaction were both impaired. The Ccz1–Mon1 complex colocalized with other fusion components on the vacuole as part of the cis-SNARE complex, and the association of the Ccz1–Mon1 complex with the vacuole appeared to be regulated by the class C Vps/HOPS complex proteins. Accordingly, we propose that the Ccz1–Mon1 complex is critical for the Ypt7-dependent tethering/docking stage leading to the formation of a trans-SNARE complex and subsequent vacuole fusion.

3.441 Active PIKfyve associates with and promotes the membrane attachment of the late endosome-to-*trans*-Golgi network transport factor Rab9 effector p40
Ikonomov, O.C. et al
J. Biol. Chem., ***278(51)***, *50863-50871 (2003)*

PIKfyve, a kinase that displays specificity for phosphatidylinositol (PtdIns), PtdIns 3-phosphate (3-P), and proteins, is important in multivesicular body/late endocytic function. Enzymatically inactive PIKfyve mutants elicit enormous dilation of late endocytic structures, suggesting a role for PIKfyve in endosome-to-*trans*-Golgi network (TGN) membrane retrieval. Here we report that p40, a Rab9 effector reported previously to bind Rab9-GTP and stimulate endosome-to-TGN transport, interacts with PIKfyve as determined by yeast two-hybrid assays, glutathione *S*-transferase (GST) pull-down assays, and co-immunoprecipitation in doubly transfected HEK293 cells. The interaction engages the PIKfyve chaperonin domain and four out of the six C-terminally positioned kelch repeats in p40. Differential centrifugation in a HEK293 cell line, stably expressing PIKfyve^{WT}, showed the membrane-associated immunoreactive p40 co-sedimenting with PIKfyve in the high speed pellet (HSP) fraction. Remarkably, similar analysis in a HEK293 cell line stably expressing dominant-negative kinase-deficient PIKfyve^{K1831E} demonstrated a marked depletion of p40 from the HSP fraction. GST-p40 failed to specifically associate with the PIKfyve lipid products PtdIns 5-P and PtdIns 3,5-P_2 in a liposome binding assay but was found to be an *in vitro* substrate of the PIKfyve serine kinase activity. A band with the p40 electrophoretic mobility was found to react with a phosphoserine-specific antibody mainly in the PIKfyve^{WT}-containing fractions obtained by density gradient sedimentation of total membranes from PIKfyve^{WT}-expressing HEK293 cells. Together these results identify the Rab9 effector p40 as a PIKfyve partner and suggest that p40-PIKfyve interaction and the subsequent PIKfyve-catalyzed p40 phosphorylation anchor p40 to discrete membranes facilitating late endosome-to-TGN transport.

3.442 Generation of the β-amyloid peptide and the amyloid percursor protein C-terminal fragment γ are potentiated by FE65L1
Chang, Y. et al
J. Biol. Chem., ***278(51)***, *511000-51107 (2003)*

Members of the FE65 family of adaptor proteins, FE65, FE65L1, and FE65L2, bind the C-terminal region of the amyloid precursor protein (APP). Overexpression of FE65 and FE65L1 was previously reported to

increase the levels of α-secretase-derived APP (APPsα). Increased β-amyloid (Aβ) generation was also observed in cells showing the FE65-dependent increase in APPsα. To understand the mechanism for the observed increase in both Aβ and APPsα given that α-secretase cleavage of a single APP molecule precludes Aβ generation, we examined the effects of FE65L1 overexpression on APP C-terminal fragments (APP CTFs). Our data show that FE65L1 potentiates γ-secretase processing of APP CTFs, including the amyloidogenic CTF C99, accounting for the ability of FE65L1 to increase generation of APP C-terminal domain and Aβ40. The FE65L1 modulation of these processing events requires binding of FE65L1 to APP and APP CTFs and is not because of a direct effect on γ-secretase activity, because Notch intracellular domain generation is not altered by FE65L1. Furthermore, enhanced APP CTF processing can be detected in early endosome vesicles but not in endoplasmic reticulum or Golgi membranes, suggesting that the effects of FE65L1 occur at or near the plasma membrane. Finally, although FE65L1 increases APP C-terminal domain production, it does not mediate the APP-dependent transcriptional activation observed with FE65.

3.443 Rat liver bile acid CoA:amino acid *N*-acyltransferase: expression, characterization, and peroxisomal localization

He, D., Barnes, S. and Falany, C.N.
J. Lipid Res., ***44****, 2242-2249 (2003)*

Bile acid CoA:amino acid *N*-acyltransferase (BAT) is responsible for the amidation of bile acids with the amino acids taurine and glycine. Rat liver BAT (rBAT) cDNA was isolated from a rat liver λZAP cDNA library and expressed in Sf9 insect cells using a baculoviral vector. rBAT displayed 65% amino acid sequence homology with human BAT (hBAT) and 85% homology with mouse BAT (mBAT). Similar to hBAT, expressed rBAT was capable of forming both taurine and glycine conjugates with cholyl-CoA. mBAT, which is highly homologous to rBAT, forms only taurine conjugated bile acids (**Falany, C. N., H. Fortinberry, E. H. Leiter, and S. Barnes.** 1997. Cloning and expression of mouse liver bile acid CoA: Amino acid N-acyltransferase. *J. Lipid Res.* **38:** 86–95). Immunoblot analysis of rat tissues detected rBAT only in rat liver cytosol following homogenization and ultracentrifugation. Subcellular localization of rBAT detected activity and immunoreactive protein in both cytosol and isolated peroxisomes. Rat bile acid CoA ligase (rBAL), the enzyme responsible for the formation of bile acid CoA esters, was detected only in rat liver microsomes. Treatment of rats with clofibrate, a known peroxisomal proliferator, significantly induced rBAT activity, message, and immunoreactive protein in rat liver. Peroxisomal membrane protein-70, a marker for peroxisomes, was also induced by clofibrate, whereas rBAL activity and protein amount were not affected.
In summary, rBAT is capable of forming both taurine and glycine bile acid conjugates and the enzyme is localized primarily in peroxisomes in rat liver.

3.444 Activation of nuclear factor-κB p50 homodimer/Bcl-3 complexes in nasapharyngeal carcinoma

Thornburg, N.J., oathmanathan, R. and Raab-Traub, N.
Cancer. Res., ***63****, 8293-8301 (2003)*

EBV latent infection is associated with the development of lymphoid and epithelial malignancies such as nasopharyngeal carcinoma (NPC). The EBV latent membrane protein 1 (LMP1) acts as a constitutively active tumor necrosis factor receptor and activates cellular signaling pathways such as c-Jun-NH_2-terminal kinase, cdc42, Akt, and nuclear factor (NF)-κB. In epithelial cells, two regions of LMP1 induce specific forms of NF-κB. COOH-terminal activating region 2 only activates p52/p65 dimers, whereas COOH-terminal activating region 1 activates p50/p50, p50/p52, and p52/p65 dimers and also uniquely up-regulates the epidermal growth factor receptor (EGFR) at the mRNA level. Deregulation of specific NF-κB members is associated with the development of many cancers. In this study, the status of NF-κB activation was investigated in NPC to determine which NF-κB dimers may contribute to the development of NPC. Electrophoretic mobility shift assay, immunoblot, ELISA, and immunohistochemistry data demonstrate that in NPC, NF-κB p50 homodimers are specifically activated, and this activation is not dependent on LMP1 expression. Coimmunoprecipitation assays indicate that homodimers are bound to the transcriptional coactivator Bcl-3, and chromatin immunoprecipitation indicates that this complex is bound to NF-κB consensus motifs within the *egfr* promoter in NPC. The discrete yet striking NF-κB p50 activation in NPC suggests that p50/p50 homodimers may be important factors in the development of NPC and may contribute to oncogenesis through transcriptional up-regulation of target genes through their interaction with Bcl-3.

3.445 Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of β-catenin, and enhanced tumor cell invasion
Lu, Z., Ghosh, S., Wang, Z. and Hunter, T.
*Cancer Cell, **4**, 499-515 (2003)*

EGF receptor (EGFR) overexpression correlates with metastasis in a variety of carcinomas, but the underlying mechanisms are poorly understood. We demonstrated that EGF disrupted cell-cell adhesion and caused epithelial-to-mesenchymal transition (EMT) in human tumor cells overexpressing EGFR, and also induced caveolin-dependent endocytosis of E-cadherin, a cell-cell adhesion protein. Chronic EGF treatment resulted in transcriptional downregulation of caveolin-1 and induction of the transcriptional repressor Snail, correlating with downregulation of E-cadherin expression. Caveolin-1 downregulation enhanced β-catenin-TCF/LEF-1 transcriptional activity in a GSK-3β-independent manner. Antisense RNA-mediated reduction of caveolin-1 expression in EGFR-overexpressing tumor cells recapitulated these EGF-induced effects and enhanced invasion into collagen gels. We propose that EGF-induced negative regulation of caveolin-1 plays a central role in the complex cellular changes leading to metastasis.

3.446 Src signaling links mediators of inflammation to Cx43 gap junction channels in primary and transformed CFTR-expressing airway cells
Huang, S. et al
*Cell Comm. and Aadhesion, **10**, 279-285 (2003)*

Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) is associated with recurrent pulmonary infections and inflammation. We previously reported that tumor necrosis factor (TNF)-f decreases gap junction connectivity in cell lines derived from the airway epithelium of non-cystic fibrosis (non-CF) subjects, a mechanism that was defective in cells derived from CF patients, and identified the tyrosine kinase c-Src as a possible bridge between TNF-f and Cx43. To examine whether this modulation also takes place in primary epithelial cells, the functional expression of Cx43 was studied in non-CF and CF airway cells, obtained from surgical polypectomies and turbinectomies, which were grown either on culture dishes or permeable filters. Expression of Cx43 was detected by immunofluorescence on cells grown under both culture conditions. Non-CF and CF airway cells also showed intercellular diffusion of Lucifer Yellow. Dye coupling was rapidly abolished in non-CF cells in the presence of TNF-f, lipopolysaccharide and lysophosphatidic acid, and could be prevented by tyrphostin47, an inhibitor of Src tyrosine kinases. This down-regulation, however, was not detected in CF airway cells. These data indicate that CFTR dysfunction is associated with altered Src signaling, resulting in the persistence of gap junction connectivity in primary and transformed CF airway cells.

3.447 Large clusters of α7-containing nicotinic acetylcholine receptors on chick spinal cord neurons
Roth, A.L. and Berg, D.K.
*J. Comp. Neurol., **465**, 195-204 (2003)*

Nicotinic acetylcholine receptors containing the α7 gene product are widely expressed in the nervous system and have high calcium permeabilities that allow them to influence numerous calcium-dependent processes. Though often found at presynaptic locations, where they enhance transmitter release, the receptors can also occupy postsynaptic sites. Highest levels have been reported for chick ciliary ganglion neurons, where the postsynaptic receptors are concentrated on somatic spines arranged in clumps and appear as large receptor clusters. We show here that subpopulations of chick spinal cord neurons also express high levels of α7-containing receptors and arrange them in large clusters. The populations include peripheral motoneurons, presumptive preganglionic neurons, neurons adjacent to the lateral motor column, and possible interneurons in the ventral horn. In many cases, the receptor clusters codistribute with filamentous actin, as do clusters on ciliary ganglion neurons, where the actin represents a somatic spine constituent. In other respects, the spinal cord clusters differ. Those on motoneurons codistribute with the actin-associated component drebrin, as do the clusters on ciliary ganglion neurons, but the clusters on preganglionic neurons do not. Preganglionic neurons do, however, stain for lipid raft components as found for ciliary ganglion neurons, where the rafts embed the receptor-enriched spines. The results demonstrate that CNS neurons can configure α7-containing nicotinic receptors into large clusters but also suggest that the clusters are not likely to reflect a common molecular substructure on all neurons.

3.448 Lipid rafts: bringing order to chaos
Pike, L.J.
*J. Lipid Res., **44**, 655-667 (2003)*

Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. They exist as distinct liquid-ordered regions of the membrane that are resistant to extraction with nonionic detergents. Rafts appear to be small in size, but may constitute a relatively large fraction of the plasma membrane. While rafts have a distinctive protein and lipid composition, all rafts do not appear to be identical in terms of either the proteins or the lipids that they contain. A variety of proteins, especially those involved in cell signaling, have been shown to partition into lipid rafts. As a result, lipid rafts are thought to be involved in the regulation of signal transduction. Experimental evidence suggests that there are probably several different mechanisms through which rafts control cell signaling. For example, rafts may contain incomplete signaling pathways that are activated when a receptor or other required molecule is recruited into the raft. Rafts may also be important in limiting signaling, either by physical sequestration of signaling components to block nonspecific interactions, or by suppressing the intrinsic activity of signaling proteins present within rafts.
This review provides an overview of the physical characteristics of lipid rafts and summarizes studies that have helped to elucidate the role of lipid rafts in signaling via receptor tyrosine kinases and G protein-coupled receptors.

3.449 Role of nephrin in proteinuric renal diseases
Salant, D.J. and Topham, P.S.
Immunopathol., ***24***, *423-439 (2003)*

No abstract available

3.450 Transport of plasma membrane-derived cholesterol and the function of Niemann-Pick C1 protein
Wiegand, V., Chang, T-Y., StraussIII, J.F., Fahrenholz, F. and Gimpl, G.
FASEB J., ***17(6)***, *782-784 (2003)*

To visualize the intracellular transport of plasma membrane-derived cholesterol under physiological and pathophysiological conditions, a novel fluorescent cholesterol analog, 6-dansyl cholestanol (DChol), has been synthesized. We present several lines of evidence that DChol mimics cholesterol. The cholesterol probe could be efficiently incorporated into the plasma membrane via cyclodextrin-donor complexes. The itinerary of DChol from the plasma membrane to the cell was studied to determine its dependence on the function of Niemann-Pick C1 (NPC) protein. In all cells, DChol moved from the plasma membrane to the endoplasmic reticulum. Its further transport to the Golgi complex was observed but with marked differences among various cell lines. DChol was finally transported to small (~0.5 μm diameter) lipid droplets, a process that required functional acyl-CoA:cholesterol acyltransferase. In human NPC fibroblasts, NPC-like cells, or in cells mimicking the NPC phenotype, DChol was found in enlarged (>1 μm diameter) droplets. When the NPC-phenotype was corrected by transfection with NPC1, DChol was again found in small-sized droplets. Our data show that NPC1 has an essential role in the distribution of plasma membrane-derived cholesterol by maintaining the small size of cholesterol-containing lipid droplets in the cell.

3.451 Insights into the Association of Fc‘RII and TCR with Detergent-Resistant Membrane Domains: Isolation of the Domains in Detergent-Free Density Gradients Facilitates Membrane Fragment Reconstitution
Korzeniowaski, M., Kwiatkowska, K. and Sobota, A.
Biochemistry, ***42***, *5358-5367 (2003)*

Plasma membrane rafts are routinely isolated as detergent-resistant membranes (DRMs) floating in detergent-free density gradients. Here we show that both the presence and exclusion of TX-100 during the density gradient fractionation have profound effects on the location of Fc‘RII and TCR in DRM fractions. The presence of TX-100 during fractionation promoted solubilization of non-cross-linked Fc‘RII when the receptor was insufficiently dissolved upon cell lysis. In the detergent-supplemented gradients, TX-100 micelles floated, further enhancing dissociation of Fc‘RII and TCR from DRMs and promoting a shift of the receptors toward higher-density fractions. Hence, fractionation of cell lysates over the detergent-containing gradients enables isolation of DRMs devoid of weakly associated proteins, like nonactivated Fc ‘RII and TCR. On the other hand, in a detergent-free gradient, non-cross-linked Fc‘RII, fully soluble in 0.2% TX-100, was recovered in DRM fractions. Moreover, employment of the TX-100-free gradient for refractionation of intermediate-density fractions, derived from detergent-supplemented gradients and containing Fc‘RII and TCR, resulted in flotation of the receptors to buoyant fractions. An analysis of the

TX-100 concentration revealed that after fractionation of 0.2% TX-100 cell lysates in the absence of detergent, the level of TX-100 in DRM fractions was reduced to 0.01%, below the critical micelle concentration. Therefore, fractionation of detergent cell lysates over detergent-free gradients can mimic conditions for a membrane reconstitution, evoking association of a distinct subset of membrane proteins, including FcγRII and TCR, with DRMs.

3.452 Engineering of technetium-99m-binding artificial receptors for imaging gene expression
Simonova, M., Shtanko, O., Serrgeyev, N., Weissleder, R. and Bogdanov Jr, A.
*J. Gene Med., **5(12)**, 1956-1066 (2003)*

Background
Optimization of gene therapy protocols requires accurate and non-invasive quantification of vector delivery and gene expression. To facilitate non-invasive imaging of gene expression, we have genetically engineered 'artificial receptors', i.e. membrane proteins that bind 99mTc-oxotechnetate (99mTcOT) via transchelation from a complex with glucoheptonate. The latter is a component of a widely used clinical imaging kit.
Methods
The engineered marker proteins were designed as type I and II membrane proteins and consisted of (1) an 99mTcOT-binding domain, metallothionein (MT), and (2) a membrane-anchoring domain. Engineered constructs were used for transfection of COS-1 and 293 cells; the expression of mRNA was verified by RT-PCR.
Results
Immunofluorescent analysis, cell fractionation and immunoblotting revealed expression of marker proteins on plasma membrane. Transfection of cells resulted in strong positive staining of plasma membrane with anti-His-tag antibodies. Scintigraphic imaging in vitro confirmed the ability of transfected cells to bind 99mTcOT. The fraction of bound radioactivity reached a peak (3.53%) when 0.93 MBq 99mTcOT was added to transfected COS-1 cells. The experiment-to-control signal ratio was equal to 32 at the same added dose.
Conclusions
(1) Both types of engineered 'artificial receptors' were expressed on the surface of eukaryotic cells; (2) marker proteins were functional in binding 99mTcOT; and (3) type II membrane proteins were more efficient in binding 99mTcOT than type I proteins. We anticipate that the developed approach could be useful for 'tagging' transfected cells with 99mTcOT enabling imaging of tracking in vivo transduced cells or cell therapies.

3.453 p53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage, and oxidative and excitotoxic insults
Gilman, C.P., Chan, S.L., Guo, Z., Zhu, X., Greig, N. and mattson, M:P.
*NeuroMol. Med., **3(3)**, 159-172 (2003)*

A form of programmed cell-death called apoptosis occurs in neurons during development of the nervous system, and may also occur in a variety of neuropathological conditions. Here we present evidence obtained in studies of adult mice and neuronal cell cultures showing that p53 protein is present in synapses where its level and amount of phosphorylation are increased following exposure of the cells to the DNA-damaging agent etoposide. We also show that levels of active p53 increase in isolated cortical synaptosomes exposed to oxidative and excitotoxic insults. Increased levels of p53 also precede loss of synapsin I immunoreactive terminals in cultured hippocampal neurons exposed to etoposide. Synaptosomes from p53-deficient mice exhibit increased resistance to oxidative and excitotoxic insults as indicated by stabilization of mitochondrial membrane potential and decreased production of reactive oxygen species. Finally, we show that a synthetic inhibitor of p53 (PFT-α) protects synaptosomes from wild-type mice against oxidative and excitotoxic injuries, and preserves presynaptic terminals in cultured hippocampal neurons exposed to etoposide. Collectively, these findings provide the first evidence for a local transcription-independent action of p53 in synapses, and suggest that such a local action of p53 may contribute to the dysfunction and degeneration of synapses that occurs in various neurodegenerative disorders.

3.454 Latent infection membrane protein transmembrane FWLY is critical for intermolecular interaction, raft localization, and signaling
Yasui, T., Luftig, M., Soni, V. and Kieff, E.
*PNAS, **101(1)**, 278-283 (2004)*

Relatively little is known about the biochemical mechanisms through which the Epstein-Barr virus latent infection integral membrane protein 1 (LMP1) transmembrane domains cause constitutive LMP1 aggregation and continuous cytoplasmic C terminus-mediated signal transduction. We now evaluate the role of the three consecutive LMP1 hydrophobic transmembrane pairs, transmembrane domains (TM)1-2, TM3-4, and TM5-6, in intermolecular aggregation and NF-κB activation. LMP1TM1-2 enabled ≈40% of wild-type LMP1 cytoplasmic domain-mediated NF-κB activation, whereas TM3-4 or TM5-6 assayed in parallel had almost no effect independent of LMP1TM1-2. Alanine mutagenesis of conserved residues in LMP1TM1-2 identified $FWLY_{38-41}$ to be critical for LMP1TM1-2 intermolecular association with LMP1TM3-6. Further, in contrast to wild-type LMP1, LMP1 with $FWLY_{38-41}$ mutated to $AALA_{38-41}$ did not (*i*) significantly partition to lipid Rafts or Barges and effectively intermolecularly associate, (*ii*) enable cytoplasmic C terminus engagement of tumor necrosis factor receptor-associated factor 3, (*iii*) activate NF-κB, and thereby (*iv*) induce tumor necrosis factor receptor-associated factor 1 expression. Other LMP1 intermolecular associations were observed that involved LMP1TM1-2/LMP1TM1-2 or LMP1TM3-4/LMP1TM3-6 interactions; these probably also contribute to LMP1 aggregation. Because $FWLY_{38-41}$ was essential for LMP1-mediated signal transduction, and LMP1 activation of NF-κB is essential for proliferating B lymphocyte survival, inhibition of $LMP1FWLY_{41}$-mediated LMP1/LMP1 intermolecular interactions is an attractive therapeutic target.

3.455 Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells
Hilenski, L.L., Clempus, R.E., Quinn, M.T., Lambeth, J.D. and Griendling, K.K.
Arterioscler. Thromb. Vasc. Biol., ***24***, *1-8 (2004)*

Objective--Reactive oxygen species (ROS) that act as signaling molecules in vascular smooth muscle cells (VSMC) and contribute to growth, hypertrophy, and migration in atherogenesis are produced by multi-subunit NAD(P)H oxidases. Nox1 and Nox4, two homologues to the phagocytic NAD(P)H subunit $gp91^{phox}$, both generate ROS in VSMC but differ in their response to growth factors. We hypothesize that the opposing functions of Nox1 and Nox4 are reflected in their differential subcellular locations.
Methods and Results--We used immunofluorescence to visualize the NAD(P)H subunits Nox1, Nox4, and $p22^{phox}$ in cultured rat and human VSMC. Optical sectioning using confocal microscopy showed that Nox1 is co-localized with caveolin in punctate patches on the surface and along the cellular margins, whereas Nox4 is co-localized with vinculin in focal adhesions. These immunocytochemical distributions are supported by membrane fractionation experiments. Interestingly, $p22^{phox}$, a membrane subunit that interacts with the Nox proteins, is found in surface labeling and in focal adhesions in patterns similar to Nox1 and Nox4, respectively.
Conclusions--The differential roles of Nox1 and Nox4 in VSMC may be correlated with their differential compartmentalization in specific signaling domains in the membrane and focal adhesions.

3.456 Akt2, phosphatidylinositol 3-kinase, and PTEN are in lipid rafts of intestinal cells: role in absorption and differentiation
Li, X., et al
Gastroenterology, ***126***, *122-135 (2004)*

Background & Aims: In intestinal Na absorptive cells, phosphatidylinositol 3-kinase (PI 3-K) is involved in rapid epidermal growth factor (EGF) stimulation of Na absorption by the brush border membrane (BBM) Na^+/H^+ exchanger NHE3. However, how NHE3 is regulated by the PI 3-K pathway and the role of Akt2 are poorly defined. *Methods:* The localization of Akt, PI 3-K, and NHE3 was determined by either immunocytochemistry and/or membrane fractionation using OptiPrep density gradient centrifugation. *Results:* In ileum, active total Akt was present most in the villi and basal layer of the crypts, and Akt2 was mostly in villi. In villus cells, PI 3-K and Akt2 were mostly at the apical surface at which they were present partially in lipid rafts (LR). EGF increased PI 3-K and active Akt2 in ileal BBM at the same time that it increased PI 3-K-dependent trafficking of NHE3 to BBM and stimulation of Na absorption. However, Akt2 was only active in the detergent soluble (DS) pool and not LR of ileal BBM, which correlated with the presence of PTEN in LR. In Caco-2 cells, while EGF stimulated BB NHE3, Akt2 was active in both LR and DS pools. This correlated with the lack of PTEN in the LR of Caco-2 membranes. Akt2 also correlated with epithelial cell differentiation. Akt2 amount and activity were greater in differentiated than undifferentiated Caco-2 cells. *Conclusions:* These results suggest that LR may play an important role in determining the function of PI 3-K/Akt2 signaling, including stimulation of intestinal Na absorption. These results also suggest that LR-associated Akt2 may be involved in enterocyte differentiation.

3.457 Mutant presenilin (A260V) affects Rab8 in PC12D cell

Kametani, F., Usami, M., Tanaka, K., Kume, H. And Mori, H.
*Neurochem. Int., **44**, 313-320 (2004)*

Most familial early-onset Alzheimer's disease (FAD) is caused by mutations in the presenilin-1 (PS1) gene. Aβ is derived from amyloid precursor protein (APP) and an increased concentration of Aβ 42 is widely believed to be a pathological hallmark of abnormal PS function. Therefore, the interaction between PS1 and APP is a central theme in attempts to clarify the molecular mechanism of AD. To examine the effect of PS1 mutations on APP metabolism, we made PC12D cell lines that express human PS1 or mutant PS1 (A260V). In PC12D cells expressing the PS1A260V mutant, we found that Rab8, a GTPase involved in transport from the *trans*-Golgi network (TGN) to the plasma membrane (PM), was significantly reduced in PC12D cells expressing the A260V mutant and that APP C-terminal fragment (CTF), the direct precursor of Aβ, accumulated in the heavy membrane fraction including membrane vesicles involved in TGN-to-PM transport. Furthermore, the total intracellular Aβ production was reduced in these cells. Combined together, we have observed that PS1 mutation disturbs membrane vesicle transport, resulting in prolonged residence of APP CTF during TGN-to-PM transport pathway. Therefore, it is highly likely that reduction of Aβ is closely related to the retention of APP CTF during TGN-to-PM transport.

3.458 BACE1 suppression by RNA interferences in primary cortical neurons

Kao, S-C., Krichevsky, A.M., Kosik, K.S. and Tsai, L-H.
*J. Biol. Chem., **279(3)**, 1942-1949 (2004)*

Extracellular deposition of amyloid-β (Aβ) aggregates in the brain represents one of the histopathological hallmarks of Alzheimer's disease (AD). Aβ peptides are generated from proteolysis of the amyloid precursor proteins (APPs) by β- and γ-secretases. β-Secretase (BACE1) is a type I integral membrane glycoprotein that can cleave APP first to generate C-terminal 99- or 89-amino acid membrane-bound fragments containing the N terminus of Aβ peptides (βCTF). As BACE1 cleavage is an essential step for Aβ generation, it is proposed as a key therapeutic target for treating AD. In this study, we show that small interfering RNA (siRNA) specifically targeted to BACE1 can suppress BACE1 (but not BACE2) protein expression in different cell systems. Furthermore, BACE1 siRNA reduced APP βCTF and Aβ production in primary cortical neurons derived from both wild-type and transgenic mice harboring the Swedish APP mutant. The subcellular distribution of APP and presenilin-1 did not appear to differ in BACE1 suppressed cells. Importantly, pretreating neurons with BACE1 siRNA reduced the neurotoxicity induced by H_2O_2 oxidative stress. Our results indicate that BACE1 siRNA specifically impacts on β-cleavage of APP and may be a potential therapeutic approach for treating AD.

3.459 Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles

Kesty, N.C. and Kuehn, M.J.
*J. Biol. Chem., **279(3)**, 2069-2076 (2003)*

Gram-negative bacteria shed outer membrane vesicles composed of outer membrane and periplasmic components. Since vesicles from pathogenic bacteria contain virulence factors and have been shown to interact with eukaryotic cells, it has been proposed that vesicles behave as delivery vehicles. We wanted to determine whether heterologously expressed proteins would be incorporated into the membrane and lumen of vesicles and whether these altered vesicles would associate with host cells. Ail, an outer membrane adhesin/invasin from *Yersinia enterocolitica*, was detected in purified outer membrane and in vesicles from *Escherichia coli* strains DH5α, HB101, and MC4100 transformed with plasmid-encoded Ail. In vesicle-host cell co-incubation assays we found that vesicles containing Ail were internalized by eukaryotic cells, unlike vesicles without Ail. To determine whether lumenal vesicle contents could be modified and delivered to host cells, we used periplasmically expressed green fluorescent protein (GFP). GFP fused with the Tat signal sequence was secreted into the periplasm via the twin arginine transporter (Tat) in both the laboratory *E. coli* strain DH5α and the pathogenic enterotoxigenic *E. coli* ATCC strain 43886. Pronase-resistant fluorescence was detectable in vesicles from Tat-GFP-transformed strains, demonstrating that GFP was inside intact vesicles. Inclusion of GFP cargo increased vesicle density but did not result in morphological changes in vesicles. These studies are the first to demonstrate the incorporation of heterologously expressed outer membrane and periplasmic proteins into bacterial vesicles.

3.460 AHNAK interaction with the annexin 2/S100A10 complex regulates cell membrane cytoarchitecture
Benaud, C.B. et al
*J. Cell Biol., **164(1)**, 133-144 (2004)*

Remodelling of the plasma membrane cytoarchitecture is crucial for the regulation of epithelial cell adhesion and permeability. In Madin-Darby canine kidney cells, the protein AHNAK relocates from the cytosol to the cytosolic surface of the plasma membrane during the formation of cell–cell contacts and the development of epithelial polarity. This targeting is reversible and regulated by Ca^{2+}-dependent cell–cell adhesion. At the plasma membrane, AHNAK associates as a multimeric complex with actin and the annexin 2/S100A10 complex. The S100A10 subunit serves to mediate the interaction between annexin 2 and the COOH-terminal regulatory domain of AHNAK. Down-regulation of both annexin 2 and S100A10 using an annexin 2–specific small interfering RNA inhibits the association of AHNAK with plasma membrane. In Madin-Darby canine kidney cells, down-regulation of AHNAK using AHNAK-specific small interfering RNA prevents cortical actin cytoskeleton reorganization required to support cell height. We propose that the interaction of AHNAK with the annexin 2/S100A10 regulates cortical actin cytoskeleton organization and cell membrane cytoarchitecture.

3.461 Mechanism of recruiting Sec6/8 (exocyst) complex to the apical juntional complex during polarization of epithelial cells
Yeaman, C., Grindstaff, K.K. and Nelson, W.J.
*J. Cell Sci., **117**, 559-570 (2004)*

Sec6/8 (exocyst) complex regulates vesicle delivery and polarized membrane growth in a variety of cells, but mechanisms regulating Sec6/8 localization are unknown. In epithelial cells, Sec6/8 complex is recruited to cell-cell contacts with a mixture of junctional proteins, but then sorts out to the apex of the lateral membrane with components of tight junction and nectin complexes. Sec6/8 complex fractionates in a high molecular mass complex with tight junction proteins and a portion of E-cadherin, and co-immunoprecipitates with cell surface-labeled E-cadherin and nectin-2α. Recruitment of Sec6/8 complex to cell-cell contacts can be achieved in fibroblasts when E-cadherin and nectin-2α are co-expressed. These results support a model in which localized recruitment of Sec6/8 complex to the plasma membrane by specific cell-cell adhesion complexes defines a site for vesicle delivery and polarized membrane growth during development of epithelial cell polarity.

3.462 Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control
Molinari, M. et al
*Mol. Cell, **13,** 125-135 (2004**)***

Calreticulin and calnexin are homologous lectins that serve as molecular chaperones for glycoproteins in the endoplasmic reticulum of eukaryotic cells. Here we show that calreticulin depletion specifically accelerates the maturation of cellular and viral glycoproteins with a modest decrease in folding efficiency. Calnexin depletion prevents proper maturation of some proteins such as influenza hemagglutinin but does not interfere appreciably with the maturation of several others. A dramatic loss of stringency in the ER quality control with transport at the cell surface of misfolded glycoprotein conformers is only observed when substrate access to both calreticulin and calnexin is prevented. Although not fully interchangeable during assistance of glycoprotein folding, calreticulin and calnexin may work, independently, as efficient and crucial factors for retention in the ER of nonnative polypeptides.

3.463 Smooth muscle raft-like membranes
Baron, C.B. and Coburn, R.F.
*J. Lipid Res.,**45**, 41-53 (2004)*

We developed a method for extracting raft-like, liquid-ordered membranes from the particulate fraction prepared from porcine trachealis smooth muscle. This fraction, which contains most of the plasma membrane in this tissue, was homogenized in the presence of cold 0.5% Triton X-100. After centrifugation, membranes containing high contents of sphingomyelin (SM) and cholesterol and low phosphatidylcholine (PC) contents remained in the pellet. Thirty-five millimolar octyl glucoside (OG) extracted 75% of these membranes from the Triton X-100-resistant pellet. These membranes had low buoyant densities and accounted for 28% of the particulate fraction lipid. Their lipid composition, 22% SM, 60% cholesterol,

11% phosphatidylethanolamine, 8% PC, <1% phosphatidylinositol, and coisolation with 5'-nucleotidase and caveolin-1 suggest that they are liquid-ordered membranes. We compared characteristics of OG and Triton X-100 extractions of the particulate fraction. In contrast to Triton X-100 extractions, membranes released from the particulate fraction by OG were mainly collected in low buoyant fractions at densities ranging from 1.05 to 1.11 g/ml and had phospholipid and cholesterol contents consistent with a mixture of liquid-ordered and liquid-disordered membranes.

3.464 The EF-hand Ca^{2+}-binding protein p22 plays a role in microtubule and endoplasmic reticulum organization and dynamics with distinct Ca^{2+}-binding requirements
Andrade, j., Zhao, H., Titus, B., Pearce, T. and Barroso, M.
*Mol. Biol. Cell, **15**, 481-496 (2004)*

We have reported that p22, an *N*-myristoylated EF-hand Ca^{2+}-binding protein, associates with microtubules and plays a role in membrane trafficking. Here, we show that p22 also associates with membranes of the early secretory pathway membranes, in particular endoplasmic reticulum (ER). On binding of Ca^{2+}, p22's ability to associate with membranes increases in an *N*-myristoylation-dependent manner, which is suggestive of a nonclassical Ca^{2+}-myristoyl switch mechanism. To address the intracellular functions of p22, a digitonin-based "bulk microinjection" assay was developed to load cells with anti-p22, wild-type, or mutant p22 proteins. Antibodies against a p22 peptide induce microtubule depolymerization and ER fragmentation; this antibody-mediated effect is overcome by preincubation with the respective p22 peptide. In contrast, *N*-myristoylated p22 induces the formation of microtubule bundles, the accumulation of ER structures along the bundles as well as an increase in ER network formation. An *N*-myristoylated Ca^{2+}-binding p22 mutant, which is unable to undergo Ca^{2+}-mediated conformational changes, induces microtubule bundling and accumulation of ER structures along the bundles but does not increase ER network formation. Together, these data strongly suggest that p22 modulates the organization and dynamics of microtubule cytoskeleton in a Ca^{2+}-independent manner and affects ER network assembly in a Ca^{2+}-dependent manner.

3.465 Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death
Muppidi, J. and Siegel, R.M.
*Nature Immunol., **5(2)**, 182-189 (2004)*

Clonotypic elimination of activated T cells through Fas–Fas ligand (CD95–CD95L) interactions is one mechanism of peripheral self-tolerance. T cell receptor (TCR) stimuli trigger FasL synthesis but also sensitize activated T cells to Fas-mediated apoptosis through an unknown mechanism. Here we show that TCR restimulation of activated human $CD4^+$ T cells resulted in Fas translocation into lipid raft microdomains before binding FasL, rendering these cells sensitive to apoptosis after stimulation with bivalent antibody or FasL. Disruption of lipid rafts reduced sensitivity to Fas-mediated apoptosis after TCR restimulation. Thus, the redistribution of Fas and other tumor necrosis factor family receptors into and out of lipid rafts may dynamically regulate the efficiency and outcomes of signaling by these receptors.

3.466 A novel fluorescence resonance energy transfer assay demonstrates that the human immunodeficiency virus type I $Pr55^{Gag}$ I domain mediates Gag-Gag interactions
Derdowski, A., Ding, L. and Spearman, P.
*J.Virol., **78(3)**, 1230-1242 (2004)*

Human immunodeficiency virus type 1 (HIV-1) assembly takes place at the plasma membrane of cells and is directed by the $Pr55^{Gag}$ polyprotein (Gag). One of the essential steps in the assembly process is the multimerization of Gag. We have developed a novel fluorescence resonance energy transfer (FRET) assay for the detection of protein-protein interactions between Gag molecules. We demonstrate that Gag multimerization takes place primarily on cellular membranes, with the majority of these interactions occurring on the plasma membrane. However, distinct sites of Gag-Gag interaction are also present at punctate intracellular locations. The I domain is a functional assembly domain within the nucleocapsid region of Gag that affects particle density, the subcellular localization of Gag, and the formation of detergent-resistant Gag protein complexes. Results from this study provide evidence that the I domain mediates Gag-Gag interactions. Using Gag-fluorescent protein fusion constructs that were previously shown to define the minimal I domain within HIV-1 $Pr55^{Gag}$, we show by FRET techniques that protein-protein interactions are greatly diminished when Gag proteins lacking the I domain are expressed. Gag-Tsg101 interactions are also seen in living cells and result in a shift of Tsg101 to the plasma membrane. The results within this study provide direct evidence that the I domain mediates protein-protein interactions

between Gag molecules. Furthermore, this study establishes FRET as a powerful tool for the detection of protein-protein interactions involved in retrovirus assembly.

3.467 The K1 protein of Kaposi's sarcoma-associated herpesvirus activates the Akt signaling pathway
Tomlinson, C.C. and Damania, B.
J. Virol., ***78(4)****, 1918-1927 (2004)*

Kaposi's sarcoma-associated herpesvirus (KSHV) has been implicated in Kaposi's sarcoma, as well as in primary effusion lymphoma and multicentric Castleman's disease. The K1 protein of KSHV has been shown to induce cellular transformation and focus formation and to deregulate B-lymphocyte signaling pathways by functionally mimicking the activated B-cell receptor complex. Here we show that expression of K1 in B lymphocytes targets the phosphatidylinositol-3 kinase pathway, leading to the activation of the Akt kinase and the inhibition of the phosphatase PTEN. We also demonstrate that activation of Akt by the K1 protein leads to the phosphorylation and inhibition of members of the forkhead (FKHR) transcription factor family, which are key regulators of cell cycle progression and apoptosis. We demonstrate that K1 can inhibit apoptosis induced by the FKHR proteins and by stimulation of the Fas receptor. Our observations suggest that the K1 viral protein promotes cell survival pathways and may contribute to KSHV pathogenesis by preventing virally infected cells from undergoing apoptosis prematurely.

3.468 Rap1 up-regulation and activation on plasma membrane regulates T cell adhesion
Bivona, T.G. et al
J. Cell Biol., ***164(3)****, 461-470 (2004)*

Rap1 and Ras are closely related GTPases that share some effectors but have distinct functions. We studied the subcellular localization of Rap1 and its sites of activation in living cells. Both GFP-tagged Rap1 and endogenous Rap1 were localized to the plasma membrane (PM) and endosomes. The PM association of GFP-Rap1 was dependent on GTP binding, and GFP-Rap1 was rapidly up-regulated on this compartment in response to mitogens, a process blocked by inhibitors of endosome recycling. A novel fluorescent probe for GTP-bound Rap1 revealed that this GTPase was transiently activated only on the PM of both fibroblasts and T cells. Activation on the PM was blocked by inhibitors of endosome recycling. Moreover, inhibition of endosome recycling blocked the ability of Rap1 to promote integrin-mediated adhesion of T cells. Thus, unlike Ras, the membrane localizations of Rap1 are dynamically regulated, and the PM is the principle platform from which Rap1 signaling emanates. These observations may explain some of the biological differences between these GTPases.

3.469 The Arf activator Gea2p and P-type ATPase Drs2p interact at the Golgi in *Saccharomyces cerevisiae*
Chantalai, S.C. et al
J. Cell Sci., ***117****, 711-722 (2004)*

Arf GTPases regulate both the morphological and protein sorting events that are essential for membrane trafficking. Guanine nucleotide exchange factors (GEFs) specific for Arf proteins determine when and where Arf GTPases will be activated in cells. The yeast Gea2p Arf GEF is a member of an evolutionarily conserved family of high molecular mass Arf GEFs that are peripherally associated with membranes. Nothing is known about how these proteins are localized to membranes, and few direct binding partners have been identified. In yeast, Gea2p has been implicated in trafficking through the Golgi apparatus and in maintaining Golgi structure. A major function of the Golgi apparatus is the packaging of cargo into secretory granules or vesicles. This process occurs through a series of membrane transformation events starting with fenestration of a saccular membrane, and subsequent remodeling of the fenestrated membrane into a mesh-like tubular network. Concentration of secretory cargo into nodes of the tubular network leads to enlargement of the nodes, which correspond to forming vesicles/granules, and thinning of the surrounding tubules. The tubules eventually break to release the secretory vesicles/granules into the cytoplasm. This process is highly conserved at the morphological level from yeast to mammalian cells. Drs2p, a multi-span transmembrane domain protein and putative aminophospholipid translocase, is required for the formation of a class of secretory granules/vesicles in yeast. Here we show that Drs2p interacts directly with Gea2p, both in vitro and in vivo. We mapped the domain of interaction of Drs2p to a 20-amino-acid region of the C-terminal cytoplasmic tail of the protein, adjacent to a region essential for Drs2p function. Mutations in Gea2p that abolish interaction with Drs2p are clustered in the C-terminal

third of the Sec7 domain, and are important for Gea2p function. We characterize one such mutant that has a thermosensitive phenotype, and show that it has morphological defects along the secretory pathway in the formation of secretory granules/vesicles.

3.470 **The matrix protein of Marburg virus is transported to the plasma membrane along cellular membranes: exploiting the retrograde late endosomal pathway**
Kolesnikova, L., Bamberg, S., Berghöfer, B. and Becker, S.
J. Virol., ***78(5)***, *2382-2393 (2004)*

VP40, the matrix protein of Marburg virus, is a peripheral membrane protein that has been shown to associate with membranes of multivesicular bodies (MVBs) (L. Kolesnikova, H. Bugany, H.-D. Klenk, and S. Becker, J. Virol. **76:**1825-1838, 2002). The present study revealed that VP40 is bound to cellular membranes rapidly after synthesis. Time course studies were performed to trace the distribution of VP40 during the course of expression. First, VP40 was homogenously distributed throughout the cytoplasm, although the majority of protein (70%) was already membrane associated. Next, VP40 accumulated in MVBs and in tubular protrusions emerging from MVBs. Finally, VP40 appeared in a patch-like pattern beneath the plasma membrane. These morphological results were supported by **iodixanol** density gradient analyses. The majority of VP40-positive membranes were first detected comigrating with small vesicles. VP40 was then shifted to fractions containing endosomal marker proteins, and later, to fractions containing plasma membrane marker proteins. Blocking of protein synthesis by use of cycloheximide at the time when VP40 was mainly associated with the small vesicles did not prevent the redistribution of VP40 to the late endosomes and further to the plasma membrane. The inhibition of intracellular vesicular trafficking by monensin significantly reduced the appearance of VP40 at the plasma membrane. In conclusion, we suggest that the transport of the Marburg virus matrix protein VP40 involves its accumulation in MVBs followed by the redistribution of VP40-enriched membrane clusters to the plasma membrane.

3.471 **Bicarbonate-responsive “soluble” adenylyl cyclase defines a nuclear camp microdomain**
Zippin, J.H. et al
J. Cell Biol., ***164(4)***, *527-534 (2004)*

Bicarbonate-responsive "soluble" adenylyl cyclase resides, in part, inside the mammalian cell nucleus where it stimulates the activity of nuclear protein kinase A to phosphorylate the cAMP response element binding protein (CREB). The existence of this complete and functional, nuclear-localized cAMP pathway establishes that cAMP signals in intracellular microdomains and identifies an alternate pathway leading to CREB activation.

3.472 **Site of docking and fusion of insulin secretory granules in live MIN6 β cells analyzed by TAT-conjugated anti-syntaxin 1 antibody and total internal reflection fluorescence microscopy**
Ohara-Imaizumi, M. et al
J. Biol. Chem., ***279(9)***, *8403-8408 (2004)*

To determine the site of insulin exocytosis in the pancreatic βcell plasma membrane, we analyzed the interaction between the docking/fusion of green fluorescent protein-tagged insulin granules and syntaxin 1 labeled by TAT-conjugated Cy3-labeled antibody (Ab) using total internal reflection fluorescence microscopy (TIRFM). Monoclonal Ab against syntaxin 1 was labeled with Cy3 then conjugated with the protein transduction domain of HIV-1 TAT. TAT-conjugated Cy3-labeled anti-syntaxin 1 Ab was transduced rapidly into the subplasmalemmal region in live MIN6 βcells, which enabled us to observe the spatial organization and distribution of endogenous syntaxin 1. TIRFM imaging revealed that syntaxin 1 is distributed in numerous separate clusters in the intact plasma membrane, where insulin secretory granules were docked preferentially to the sites of syntaxin 1 clusters, colocalizing with synaptosomal-associated protein of 25 kDa (SNAP-25) clusters. TIRFM imaging analysis of the motion of single insulin granules demonstrated that the fusion of insulin secretory granules stimulated by 50 mM KCl occurred exclusively at the sites of the syntaxin 1 clusters. Cholesterol depletion by methyl-β-cyclodextrin treatment, in which the syntaxin 1 clusters were disintegrated, decreased the number of docked insulin granules, and, eventually the number of fusion events was significantly reduced. Our results indicate that 1) insulin exocytosis occurs at the site of syntaxin 1 clusters; 2) syntaxin 1 clusters are essential for the docking and fusion of insulin granules in MIN6 βcells; and 3) the sites of syntaxin 1 clusters are distinct from flotillin-1 lipid rafts.

3.473 Sphingolipid-cholesterol domains (lipid rafts) in normal human and dog thyroid follicular cells are not involved in thyrotropin receptor signaling
Costa, M.J. et al
*Endocrinology, **145(3)**, 1464-1472 (2004)*

Partition of signaling molecules in sphingolipid-cholesterol-enriched membrane domains, among which are the caveolae, may contribute to signal transduction efficiency. In normal thyroid, nothing is known about a putative TSH/cAMP cascade compartmentation in caveolae or other sphingolipid-cholesterol-enriched membrane domains. In this study we show for the first time that caveolae are present in the apical membrane of dog and human thyrocytes: caveolin-1 mRNA presence is demonstrated by Northern blotting in primary cultures and that of the caveolin-1 protein by immunohistochemistry performed on human thyroid tissue. The TSH receptor located in the basal membrane can therefore not be located in caveolae. We demonstrate for the first time by biochemical methods the existence of sphingolipid-cholesterol-enriched domains in human and dog thyroid follicular cells that contain caveolin, flotillin-2, and the insulin receptor. We assessed a possible sphingolipid-cholesterol-enriched domains compartmentation of the TSH receptor and the α- subunit of the heterotrimeric G_s and G_q proteins using two approaches: Western blotting on detergent-resistant membranes isolated from thyrocytes in primary cultures and the influence of 10 mM methyl-ß-cyclodextrin, a cholesterol chelator, on basal and stimulated cAMP accumulation in intact thyrocytes. The results from both types of experiments strongly suggest that the TSH/cAMP cascade in thyroid cells is not associated with sphingolipid-cholesterol-enriched membrane domains.

3.474 Characterization of the Lassa virus matrix protein Z: electron microscopic study of virus-like particles and interaction with the nucleoprotein (NP)
Eichler, R. et al
*Virus Res., **100**, 249-255 (2004)*

Lassa virus is the causative agent of a hemorrhagic fever endemic in west Africa. The RNA genome of Lassa virus encodes the glycoprotein precursor GP-C, a nucleoprotein (NP), the viral polymerase L and a small protein Z (11 kDa). Here, we analyze the role of Z protein for virus maturation. We have recently shown that expression of Z protein in the absence of other viral proteins is sufficient for the release of enveloped Z-containing particles. In this study, we examined particles secreted into the supernatant of a stably Z protein-expressing CHO cell line by electron microscopy. The observed Z-induced virus-like particles did not significantly differ in their morphology and size from Lassa virus particles. Mutation of two proline-rich domains within Z which are known to drastically reduce the release of virus-like particles, had no effect on the cellular localization of the protein nor on its membrane-association. Furthermore, we present evidence that Z interacts with the NP. We assume that Z recruits NP to cellular membranes where virus assembly takes place. We conclude from our data that Lassa virus Z protein plays an essential role in Lassa virus maturation.

3.475 Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the *Helicobacter pylori* vacuolating cytotoxin VacA in HeLa cells
Gauthier, N.C. et al
*J. Biol. Chem., **279(10)**, 9481-9489 (2004)*

The vacuolating cytotoxin VacA is an important virulence factor of *Helicobacter pylori*. Removing glycosylphosphatidylinositol-anchored proteins (GPI-Ps) from the cell surface by phosphatidylinositol-phospholipase C or disrupting the cell actin cytoskeleton by cytochalasin D reduced VacA-induced vacuolation of cells (Ricci V., Galmiche, A., Doye, A., Necchi, V., Solcia, E., and Boquet, P. (2000) *Mol. Biol. Cell* 11, 3897-3909). Using the fluorescent dye 6-methoxy-*N*-ethylquinolinium chloride, an indicator for cytosolic chloride, we have investigated the role of either GPI-Ps or actin cytoskeleton in the activity of the selective anionic channel formed by VacA at the plasma membrane level. Removal of GPI-Ps from HeLa cell surfaces did not impair VacA localization into lipid rafts but strongly reduced VacA channel-mediated cell influx and efflux of chloride. Disruption of the actin cytoskeleton of HeLa cells by cytochalasin D did not affect VacA localization in lipid rafts but blocked VacA cell internalization and inhibited cell vacuolation while increasing the overall chloride transport by the toxin channel at the cell

surface. Specific enlargement of Rab7-positive compartments induced by VacA could be mimicked by the weak base chloroquine alone, and the vacuolating activities of either chloroquine alone or VacA were blocked with the same potency by the anion channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid shown to inhibit VacA channel activity (Tombola, F., Oregna, F., Brutsche, S., Szabò, I., Del Giudice, G., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) *FEBS Lett.* 460, 221-225). We suggest that formation of functional VacA channels at the cell surface required GPI-Ps and that endocytosis of these channels by an actin-dependent process increases the chloride content of late endosomes that accumulate weak bases, provoking their enlargement by osmotic swelling.

3.476 Human immunodeficiency virus type 1 Nef activates p21-activated kinase vis recruitment into lipid rafts

Krautkrämer, E., Giese, S.I., Gasteier, J.E., Muuranyi, W. and Fackler, O.T.
*J. Virol., **78(8)**, 4085-4097 (2004)*

The Nef protein of human immunodeficiency virus type 1 is an important factor in AIDS pathogenesis. In addition to downregulating CD4 and major histocompatibility complex class I molecules from the cell surface, as well as increasing virion infectivity, Nef triggers activation of the T-cell receptor (TCR) cascade to facilitate virus spread. Signaling pathways that are induced by Nef have been identified; however, it is unclear how and in which subcellular compartment Nef triggers signaling. Nef recruits a multiprotein complex to activate the cellular Pak kinase that mediates downstream effector functions. Since a subpopulation of Nef is present in detergent-insoluble microdomains (lipid rafts) from where physiological TCR signaling is initiated, we tested whether lipid rafts are instrumental for Nef-mediated Pak activation. In flotation analysis, Nef-associated Pak activity exclusively fractionated with lipid rafts. Activation of Pak in the presence of Nef coincided with lipid raft recruitment of the kinase, which was otherwise excluded from detergent-insoluble microdomains. Experimental solubilization of lipid rafts interfered with the association of Pak activity with Nef. To analyze the importance of the raft localization for Nef function more rigorously, we generated a palmitoylated Nef (PalmNef). PalmNef was highly enriched in lipid rafts and associated with significantly higher levels of Pak activity than Nef. Notably, activation of Pak by its physiological activators, Cdc42 and Rac, also occurred in lipid rafts and required raft integrity. Together, these data suggest that Nef induces signal transduction via the recruitment of a signaling machinery including Pak into lipid rafts, thereby mimicking a physiological cellular mechanism to initiate the TCR cascade.

3.477 Conserved "PAL" sequence in presenilins is essential for γ-secretase activity, but not required for formation or stabilization of γ-secretase complexes

Wang, J., Brunkan, A.L., Hecimovic, S., Walker, E. and Goate, A.
*Neurobiol.of Disease, **15**, 654-666 (2004)*

Generation of Aβ from the β-amyloid precursor protein (APP) requires a series of proteolytic processes, including an intramembranous cleavage catalyzed by an aspartyl protease, γ-secretase. Two aspartates in presenilins (PS) are required for γ-secretase activity (D257 and D385 of PS1), suggesting that PS may be part of this protease. Little is known concerning the importance of other sequences in PS for activity. We introduced point mutations (P433L, A434D, L435R) into a completely conserved region C-terminal to transmembrane domain eight of PS1. The P433L mutation abolished PS1 endoproteolysis as well as γ-secretase cleavage of APP and Notch in PS1/2 K/O cells. In HEK cells, expression of PS1/P433L reduced Aβ production and caused accumulation of APP C-terminal stubs. When the P433L mutation was introduced into the non-cleavable Δexon 9 (ΔE9) variant of PS1, it abolished γ-secretase cleavage of APP and Notch. The P433L holoprotein is stable and incorporated into the high molecular weight γ-secretase complex, arguing that P433 is not necessary for formation or stabilization of the γ-secretase complex. Other non-conservative mutations in the invariant $P_{433}A_{434}L_{435}$ sequence also result in a phenotype that is indistinguishable from the aspartate mutants, suggesting a direct involvement of this sequence in γ-secretase activity.

3.478 Secreted MMP9 promotes angiogenesis more efficiently than constitutive active MMP9 bound to the tumor cell surface

Mira, E. et al
*J. Cell Sci., **117**, 1847-1856 (2004)*

Association of matrix metalloprotease 9 (MMP9) to the cell membrane is considered important in tumor growth and angiogenesis. To dissect this regulatory mechanism, we generated raft and non-raft MMP9 chimeras to force membrane expression in the MCF-7 human breast carcinoma cell line. MMP9 targeting to non-raft cell surface domains rendered a constitutive active membrane MMP9 form, suggesting a contribution by the lipid environment in MMP activation. We generated human breast cancer xenograft models using MCF-7 cells overexpressing secreted and membrane-anchored MMP9. The non-raft MMP9 chimera was constitutively active at the cell membrane in xenografts, but this activation did not correlate with an increase in MMP9-induced angiogenesis. Capillary number and vessel perimeter were specifically increased only in tumors overexpressing wild-type MMP9 (the secreted form); this increase was inhibited when tumors were induced in doxycycline-treated mice. Xenografts from tumor cells overexpressing wild-type MMP9 showed increased vascular endothelial growth factor (VEGF)/VEGFR2 receptor association, which was also dependent on MMP9 activity. These observations indicate that membrane location can influence MMP9 activity in vitro and in vivo, and confirm the relevance of stromal-associated, but not tumor-bound MMP9 in mediating tumor-induced angiogenesis.

3.479 Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi
Hirose, H. et al
EMBO J., ***23***, *1267-1278 (2004)*

ZW10, a dynamitin-interacting protein associated with kinetochores, is known to participate directly in turning off of the spindle checkpoint. In the present study, we show that ZW10 is located in the endoplasmic reticulum as well as in the cytosol during interphase, and forms a subcomplex with RINT-1 (Rad50-interacting protein) and p31 in a large complex comprising syntaxin 18, an endoplasmic reticulum-localized t-SNARE implicated in membrane trafficking. Like conventional syntaxin-binding proteins, ZW10, RINT-1 and p31 dissociated from syntaxin 18 upon Mg^{2+}-ATP treatment in the presence of NSF and α-SNAP, whereas the subcomplex was not disassembled. Overexpression, microinjection and knockdown experiments revealed that ZW10 is involved in membrane trafficking between the endoplasmic reticulum and Golgi. The present results disclose an unexpected role for a spindle checkpoint protein, ZW10, during interphase.

3.480 Appropriate NR1-NR1 disulfide-linked homodimer formation is requisite for efficient expression of functional, cell surface N-methyl-D-aspartate NR1/NR2 receptors
Papadakis, M., Hawkins, L.M. and Stephenson, F.A.
J. Biol. Chem., ***279(15)***, *14703-14712 (2004)*

A c-Myc epitope-tagged *N*-methyl-D-aspartate receptor NR1-2a subunit was generated, NR1-$2a_{c\text{-}Myc}$, where the tag was inserted after amino acid 81. NR1-$2a_{c\text{-}Myc}$ /NR2A receptors when expressed in mammalian cells are not trafficked to the cell surface nor do they yield cell cytotoxicity post-transfection. NR1-$2a_{c\text{-}Myc}$ was, however, shown to assemble with NR2A subunits by immunoprecipitation and [^{3}H]MK801 radioligand binding assays. Immunoblots of cells co-transfected with wild-type NR1-2a/NR2A subunits yielded two NR1-2a immunoreactive species with molecular masses of 115 and 226 kDa. Two-dimensional electrophoresis under non-reducing and reducing conditions revealed that the 226-kDa band contained disulfide-linked NR1-2a subunits. Only the 115-kDa NR1-2a species was detected for NR1-$2a_{c\text{-}Myc}$/NR2A. The c-Myc epitope is inserted adjacent to cysteine 79 of the NR1-2a subunit; therefore, it is possible that the tag may prevent the formation of NR1 disulfide bridges. A series of cysteine →alanine NR1-2a mutants was generated, and the NR1-2a mutants were co-expressed with NR2A or NR2B subunits in mammalian cells and characterized with respect to cell surface expression, cell cytotoxicity post-transfection, co-association by immunoprecipitation, and immunoblotting following SDS-PAGE under both reducing and non-reducing conditions. When co-expressed with NR2A in mammalian cells, NR1-$2a_{C79A}$/NR2A displayed similar properties to NR1-$2a_{c\text{-}Myc}$/NR2A in that the 226-kDa NR1 immunoreactive species was not detectable, and trafficking to the cell surface was impaired compared with wild-type NR1/NR2 receptors. These results provide the first biochemical evidence for the formation of NR1-NR1 intersubunit disulfide-linked homodimers involving cysteine 79. They suggest that disulfide bridging and structural integrity within the NR1 N-terminal domain is requisite for cell surface *N*-methyl-D-aspartate receptor expression.

3.481 Gene and protein characterization of the human glutathione S-transferase kappa and evidence for a peroxisomal localization
Morel, F. et al
J. Biol. Chem., ***279(16)***, *16246-16253 (2004)*

Kappa class glutathione *S*-transferase (GST) cDNA sequences have been identified in rat, mouse, and human. In the present study, we determined the structure and chromosomal location of the human GST Kappa 1 (*hGSTK1*) gene, characterized the protein, and demonstrated its subcellular localization. The human gene spans ~5 kb, has 8 exons, and maps onto chromosome 7q34. The 5'-flanking region lacks TATA or CCAAT boxes, but there is an initiator element overlapping the transcription start site. hGSTK1 amino acid sequence showed homology to bacterial 2-hydroxychromene-2-carboxylate isomerase, an enzyme involved in naphthalene degradation pathway. hGSTK1 mRNA was expressed in all of the organs examined. Subcellular fractionation of HepG2 cells showed that the protein was located in peroxisomes and mitochondria and was not detectable in cytoplasm. The peroxisomal localization was confirmed by transfection of HepG2 cells with a plasmid coding a green fluorescent protein fused inframe to the N terminus of hGSTK1. The C terminus of hGSTK1 was essential for localization of the protein to peroxisomes, and the C-terminal sequence Ala-Arg-Leu represents a peroxisome targeting signal. This is the first time that a human GST has been found in peroxisomes, suggesting a new function for this family of enzymes.

3.482 Pen-2 is sequestered in the endoplamic reticulum and subjected to ubiquitylation and proteasome-mediated degradation in the absence of presenilin
Bergmann, A. et al
J. Biol. Chem., ***279(16)***, *16744-16753 (2004)*

The γ-secretase complex catalyzes intramembrane proteolysis of a number of transmembrane proteins, including amyloid precursor protein, Notch, ErbB4, and E-cadherin. γ-Secretase is known to contain four major protein constituents: presenilin (PS), nicastrin, Aph-1, and Pen-2, all of which are integral membrane proteins. There is increasing evidence that the formation of the complex and the stability of the individual components are tightly controlled in the cell, assuring correct composition of functional complexes. In this report, we investigate the topology, localization, and mechanism for destabilization of Pen-2 in relation to PS function. We show that PS1 regulates the subcellular localization of Pen-2: in the absence of PS, Pen-2 is sequestered in the endoplasmic reticulum (ER) and not transported to post-ER compartments, where the mature γ-secretase complexes reside. PS deficiency also leads to destabilization of Pen-2, which is alleviated by proteasome inhibitors. In keeping with this, we show that Pen-2, which adopts a hairpin structure with the N and C termini facing the luminal space, is ubiquitylated prior to degradation and presumably retrotranslocated from the ER to the cytoplasm. Collectively, our data suggest that failure to become incorporated into the γ-secretase complex leads to degradation of Pen-2 through the ER-associated degradation-proteasome pathway.

3.483 Seasonal varation in cytochrome P450 immunopositive protein levels, lipid peroxidation and genetic toxicity in digestive gland of the mussel *Mytilus edulis*
Shaw, J.P. et al
Aquatic Tox., ***67***, *325-336 (2004)*

The relationship between cytochrome P450 1A- and 2E-immunopositive proteins, lipid peroxidation and DNA strand breaks (SBs) was studied in *Mytilus edulis* digestive gland at different seasons and at different sites around the UK coast. Cytochrome P4501A (CYP1A)-immunopositive protein and DNA strand breaks were generally lowest in December but there was no correlation between PAH exposure (indicated by chemical measurement and CYP1A-immunopositive protein expression) and DNA strand breaks which was highest at the relatively non-polluted site (Port Quin). As with CYP1A, CYP2E1-immunopositive protein was maximal at most sites in May. Lipid peroxidation, in contrast, did not alter markedly throughout the year. In conclusion, DNA strand breakage was not correlated with any of the above parameters although it did correlate with "scope for growth" as did the inverse of PAH levels. The study highlights the need to establish the relative contribution of DNA damage and DNA repair processes to the production of DNA strand breaks and emphasises the need to consider seasonal variation in interpretation of biomarkers.

3.484 The 2003 ASBMB-Avanti Award in Lipids Address: applications of novel synthetic lipids to biological problems
Bittmann, R.
Chemistry & Lipids, ***129***, *111-131 (2004)*

This paper is an overview of the 2003 Avanti Award in Lipids address that was presented by Robert Bittman at the American Society for Biochemistry and Molecular Biology (ASBMB) Annual Meeting held in San Diego, CA in conjunction with meetings of five other FASEB Societies, April 15, 2003. The theme of the lecture is: "How can the chemical synthesis of unnatural lipids provide insights into problems ranging from cell biology to biophysics?" The following examples are presented: (1) novel ceramide analogs as experimental anticancer agents, (2) photoactivatable sphingosine 1-phosphate analogs as probes of protein targets of this bioactive lipid, (3) a ^{13}C-enriched cerebroside as a quantitative probe of glycosphingolipid (GSL) transbilayer distribution in bilayers with and without sphingomyelin, (4) *cis* and *trans* unsaturated sphingomyelin analogs as modulators of the existence of cholesterol-enriched microdomains (rafts) that may facilitate fusion of alphaviruses with target membranes, (5) ceramide as an indirect enhancer of the permeabilization of membranes induced by cholesterol-specific cytolysins, (6) fluorescent GSL analogs of widely disparate structure as probes of the molecular features responsible for the selective internalization of GSLs in caveolae of living mammalian cells, (7) enantiomeric lysophosphatidic acid (LPA) analogs as probes of receptor subtypes that mediate LPA signaling, and (8) phosphonocholine analogs of the antitumor ether lipid ET-18-OCH_3 as tools for discerning the primary targets that are critical for cytotoxic activity in tumor cells.

3.485 CD44 interaction with ankyrin and IP_3 receptor in lipid rafts promotes hyaluronan-mediated Ca^{2+} signaling to nitric oxide production and endothelial cell adhesion and proliferation

Singleton, P.A. and Bourguignon, L.Y.W.
Exp. Cell Res., ***295***, *102-118 (2004)*

In this study, we have showed that aortic endothelial cells (GM7372A cell line) express CD44v10 [a hyaluronan (HA) receptor], which is significantly enriched in cholesterol-containing lipid rafts (characterized as caveolin-rich plasma membrane microdomains). HA binding to CD44v10 promotes recruitment of the cytoskeletal protein, ankyrin and inositol 1,4,5-triphosphate (IP_3) receptor into cholesterol-containing lipid rafts. The ankyrin repeat domain (ARD) of ankyrin is responsible for binding IP_3 receptor to CD44v10 at lipid rafts and subsequently triggering HA/CD44v10-mediated intracellular calcium (Ca^{2+}) mobilization leading to a variety of endothelial cell functions such as nitric oxide (NO) production, cell adhesion and proliferation.
Further analyses indicate (i) disruption of lipid rafts by depleting cholesterol from the membranes of GM7372A cells (using methyl-β-cyclodextrin treatment) or (ii) interference of endogenous ankyrin binding to CD44 and IP_3 receptor using overexpression of ARD fragments (by transfecting cells with ARDcDNA) not only abolishes ankyrin/IP_3 receptor accumulation into CD44v10/cholesterol-containing lipid rafts, but also blocks HA-mediated Ca^{2+} signaling and endothelial cell functions. Taken together, our findings suggest that CD44v10 interaction with ankyrin and IP_3 receptor in cholesterol-containing lipid rafts plays an important role in regulating HA-mediated Ca^{2+} signaling and endothelial cell functions such as NO production, cell adhesion and proliferation.

3.486 Heparan sulphate proteoglycans modulate fibroblast growth factor-2 binding through a lipid-raft-mediated mechanism

Chu, C.L., Buczek-Thomas, J.A. and Nugent, M.A.
Biochem. J., ***379***, *331-341 (2004)*

We investigated how lipid raft association of HSPG (heparan sulphate proteoglycans) modulates FGF-2 (fibroblast growth factor-2/basic fibroblast growth factor) interactions with vascular smooth-muscle cells. When lipid rafts were disrupted with sterol-binding agents, methyl-alpha-cyclodextrin and filipin, FGF-2 binding to HSPG was reduced 2-5-fold, yet the amount and turnover of cell-surface HSPG were unaffected. Approx. 50-65% of bound FGF-2 was in lipid raft-associated fractions based on insolubility in unlabelled Triton X-100 and flotation in OptiPrep density gradients, and this level was increased with higher FGF-2 concentrations. Less FGF-2 (50-90%) was associated in raft fractions when cholesterol was depleted or HSPG were degraded with heparinase III. To investigate how lipid raft-HSPG interactions altered binding, we compared the rates of FGF-2 dissociation with native, MbetaCD (methyl-beta-cyclodextrin)- and filipin-treated cells. We found that FGF-2 dissociation rates were increased when lipid rafts were disrupted. These results suggest that localization of HSPG within lipid rafts creates high local concentrations of binding sites such that dissociation of FGF-2 is hindered. The localization of FGF-2 and HSPG to lipid rafts also correlated with the activation of protein kinase Calpha. Thus raft association of HSPG might create growth factor traps resulting in increased binding and signal transduction to enhance cell sensitivity.

3.487 Lipid rafts and integrin activation regulate oligodendrocyte survival
Decker, L. and ffrench-Constant, C.
J. Neurosci., ***24(15)*** *3816-3825 (2004)*

Newly formed oligodendrocytes in the CNS derive survival cues from their target axons. These cues are provided in part by laminins expressed on the axon, which are recognized by $\alpha 6\beta 1$ integrin on the oligdendrocyte and amplify platelet-derived growth factor (PDGF) signaling through the phosphatidylinositol 3'-kinase (PI3K) pathway. The $\alpha 6\beta 1$ integrin is localized in oligodendrocyte lipid rafts. We show here using the sphingolipid synthesis inhibitor fumonisin-B1 to deplete rafts that this localization is important for normal survival signaling, because depletion increases oligodendrocyte apoptosis and inhibits PI3K signaling. We have shown previously that PDGF-mediated integrin activation is an important component of oligodendrocyte proliferation signaling, and here we present evidence that a similar mechanism operates in survival signaling. Integrin activation using manganese increases raft localization and rescues the effects of both raft depletion and PDGF removal on survival and PI3K signaling. Together, these results point to an essential role for rafts in oligodendrocyte survival signaling on the basis of the provision of a favorable environment for growth factor-mediated integrin activation.

3.488 Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes
Watson, R.T., Kanzaki, M. and Pessin, J.E.
Endocrine Reviews, ***25****, 177-204 (2004)*

Since the discovery of insulin roughly 80 yr ago, much has been learned about how target cells receive, interpret, and respond to this peptide hormone. For example, we now know that insulin activates the tyrosine kinase activity of its cell surface receptor, thereby triggering intracellular signaling cascades that regulate many cellular processes. With respect to glucose homeostasis, these include the function of insulin to suppress hepatic glucose production and to increase glucose uptake in muscle and adipose tissues, the latter resulting from the translocation of the glucose transporter 4 (GLUT4) to the cell surface membrane. Although simple in broad outline, elucidating the molecular intricacies of these receptor-signaling pathways and membrane-trafficking processes continues to challenge the creative ingenuity of scientists, and many questions remain unresolved, or even perhaps unasked. The identification and functional characterization of specific molecules required for both insulin signaling and GLUT4 vesicle trafficking remain key issues in our pursuit of developing specific therapeutic agents to treat and/or prevent this debilitating disease process. To this end, the combined efforts of numerous research groups employing a range of experimental approaches has led to a clearer molecular picture of how insulin regulates the membrane trafficking of GLUT4.

3.489 Activation of the tumor suppresor merlin modulates its interaction with lipid rafts
Stickney, J.T., Bacon, W.C., Rojas, M., Ratner, N. and Ip W.
Cancer Res., ***64****, 2717-2724 (2004)*

Neurofibromatosis type 2 (NF2) is a genetic disorder characterized by bilateral schwannomas of the eighth cranial nerve. The *NF2* tumor suppressor protein, merlin, is related to the ERM (ezrin, radixin, and moesin) family of membrane/F-actin linkers. Merlin resists solubilization by the detergent Triton X-100 (TX-100), a property commonly attributed to association with the cytoskeleton. Accordingly, NF2 patient mutations that encode merlins with enhanced TX-100 solubility have been explained previously in terms of loss of cytoskeletal attachment. However, here we present data to suggest that the detergent resistance of merlin is a result of its constitutive residence in lipid rafts. Furthermore, when cells are grown to high density, merlin shifts to a more buoyant lipid raft fraction in a density gradient. This shift is mimicked in subconfluent cells treated with cytochalasin D, suggesting that the shift results from merlin dissociation from the actin cytoskeleton, but not from lipid rafts. Intramolecular NH_2- and COOH-terminal binding, which occurs when merlin transitions to the growth-suppressive form, also brings about a similar change in buoyant density. Our results suggest that constitutive residence of merlin in lipid rafts is crucial for its function and that as merlin becomes growth suppressive *in vivo*, one significant molecular event may be the loss of interaction with the actin cytoskeleton. To our knowledge, merlin is the first tumor suppressor known to reside within lipid rafts, and the significance of this finding is underscored by known loss-of-function NF2 patient mutations that encode merlins with enhanced TX-100 solubility.

3.490 **Carbachol regulation of rabbit ileal brush border Na^+-H^+ exchanger 3 (NHE3) occurs through changes in NHE3 trafficking and complex formation and is Src dependent**
Li, X. et al
J. Physiol., ***3***, *791-804 (2004)*

The epithelial brush border membrane (BBM) Na^+-H^+ exchanger 3 (NHE3) is the major transport protein responsible for ileal electroneutral Na^+ absorption. We have previously shown that ileal BBM NHE3 activity is rapidly inhibited by carbachol, an agonist that mimics cholinergic activation in digestion. In this study, we investigated the mechanisms involved in this NHE3 inhibition. Carbachol decreased the amount of ileal Na^+ absorptive cell BBM NHE3 within 10 min of exposure. Based on **OptiPrep** gradient centrifugation, carbachol increased the amount of NHE3 in early endosomes and decreased the amount of NHE3 in BBM, consistent with effects on NHE3 trafficking. The decrease in BBM NHE3 occurred in the detergent-soluble BBM fraction with no change in the amount of NHE3 in the BBM detergent-resistant membranes. The size of BBM NHE3 complexes increased in carbachol-exposed ileum, as studied with sucrose gradient centrifugation. The NHE3 complex size increased in the total BBM, but did not change in the detergent-soluble fraction. This suggests that carbachol treatment enhanced the association of proteins with NHE3 complexes specifically in the detergent-resistant fraction of ileal BBM. NHERF2, α-actinin-4 and protein kinase C were among those NHE3-associated proteins because they were more efficiently coimmunoprecipitated from total BBM after carbachol treatment. Moreover, Src was involved in the carbachol-mediated inhibition since: (1) c-Src was rapidly activated in the detergent-resistant membranes by carbachol; and (2) carbachol inhibition of ileal Na^+ absorption was completely abolished by the Src family inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2). Moreover, the carbachol-induced increase in the size of NHE3-containing complexes was reversed by PP2. These data demonstrate that regulation of NHE3 activity by carbachol can be achieved at several interrelated levels: (1) the subcellular level, at which NHE3 is rapidly endocytosed from BBM to endocytic vesicles upon treatment with carbachol; (2) multiple BBM pools, in which carbachol selectively decreases the amount of NHE3 in the BBM detergent-soluble fraction but not the detergent-resistant membrane; and (3) the molecular level, at which NHE3 complex-associated proteins can be changed upon carbachol treatment, with carbachol leading to larger BBM NHE3 complexes and increased co-IP of NHERF2 with α-actinin-4 and activated PKC. The study further describes NHE3 presence simultaneously in multiple dynamic BBM pools in which NHE3 distribution and associated proteins are altered as part of carbachol-induced and Src-mediated rapid signal transduction, which decreases the amount of BBM NHE3 and thus inhibits NHE3 activity.

3.491 **Ubiquitin-mediated targeting of a mutant plasma membrane ATPase, Pma1-7, to the endosomal/vacuolar system in yeast**
Pizzirusso, M. and Chang, A.
Mol. Biol. Cell, ***15***, *2401-2409 (2004)*

Pma1-7 is a mutant plasma membrane ATPase that is impaired in targeting to the cell surface at 37°C and is delivered instead to the endosomal/vacuolar pathway for degradation. We have proposed that Pma1-7 is a substrate for a Golgibased quality control mechanism. By contrast with wild-type Pma1, Pma1-7 is ubiquitinated. Ubiquitination and endosomal targeting of Pma1-7 is dependent on the Rsp5-Bul1-Bul2 ubiquitin ligase protein complex but not the transmembrane ubiquitin ligase Tul1. Analysis of Pma1-7 ubiquitination in mutants blocked in protein transport at various steps of the secretory pathway suggests that ubiquitination occurs after ER exit but before endosomal entry. In the absence of ubiquitination in *rsp5-1* cells, Pma1-7 is delivered to the cell surface and remains stable. Nevertheless, Pma1-7 remains impaired in association with detergent-insoluble glycolipid-enriched complexes in *rsp5-1* cells, suggesting that ubiquitination is not the cause of Pma1-7 exclusion from rafts. In *vps1* cells in which protein transport into the endosomal pathway is blocked, Pma1-7 is routed to the cell surface. On arrival at the plasma membrane in *vps1* cells, Pma1-7 remains stable and its ubiquitination disappears, suggesting deubiquitination activity at the cell surface. We suggest that Pma1-7 sorting and fate are regulated by ubiquitination.

3.492 **Rotavirus RRV associates with lipid membrane microdomains during cell entry**
Isa, P., Realpe, M., Romero, P., Lopez, S. and Arias, C.F.

*Virology, **322**, 370-381 (2004)*

Rotavirus cell entry is a multistep process, not completely understood, which requires at least four interactions between the virus and cell surface molecules. In this work, we investigated the role of the sphingolipid- and cholesterol-enriched lipid microdomains (rafts) in the entry of rotavirus strain RRV to MA104 cells. We found that ganglioside GM1, integrin subunits α2 and β3, and the heat shock cognate protein 70 (hsc70), all of which have been implicated as rotavirus receptors, are associated with TX-100 and Lubrol WX detergent-resistant membranes (DRMs). Integrin subunits α2 and β3 were found to be particularly enriched in DRMs resistant to lysis by Lubrol WX. When purified RRV particles were incubated with cells at 4 °C, about 10% of the total infectious virus was found associated with DRMs, and the DRM-associated virus increased to 37% in Lubrol-resistant membrane domains after 60-min incubation at 37 °C. The virus was excluded from DRMs if the cells were treated with methyl-β-cyclodextrin (MβCD). Immunoblot analysis of the viral proteins showed that the virus surface proteins became enriched in DRMs upon incubation at 37 °C, being almost exclusively localized in Lubrol-resistant DRMs after 60 min. These data suggest that detergent-resistant membrane domains play an important role in the cell entry of rotaviruses, which could provide a platform to facilitate the efficient interaction of the rotavirus receptors with the virus particle.

3.493 Tula hantavirus L protein is a 250 kDa perinuclear membrane-associated protein
Kukkonen, S.K.J., Vaheri, A. And Plyusin, A.
*J. Gen. Virol., **85**, 1181-1189 (2004)*

The complete open reading frame of Tula hantavirus (TULV) L RNA was cloned in three parts. The middle third (nt 2191–4344) could be expressed in *E. coli* and was used to immunize rabbits. The resultant antiserum was then used to immunoblot concentrated TULV and infected Vero E6 cells. The L protein of a hantavirus was detected, for the first time, in infected cells and was found to be expressed as a single protein with an apparent molecular mass of 250 kDa in both virions and infected cells. Using the antiserum, the expression level of the L protein was followed and image analysis of immunoblots indicated that there were 10^4 copies per cell at the peak level of expression. The antiserum was also used to detect the L protein in cell fractionation studies. In cells infected with TULV and cells expressing recombinant L, the protein pelleted with the microsomal membrane fraction. The membrane association was confirmed with membrane flotation assays. To visualize L protein localization in cells, a fusion protein of L and enhanced green fluorescent protein, L–EGFP, was expressed in Vero E6 cells with a plasmid-driven T7 expression system. L–EGFP localized in the perinuclear region where it had partial co-localization with the Golgi matrix protein GM130 and the TULV nucleocapsid protein.

3.494 Cholesterol-independent interactions with CD47 enhance $\alpha_v\beta_3$ activity
McDonald, J.F., Zheleznyak, A. and Frazier, W.A.
*J. Biol. Chem., **279(17)**, 17301-17311 (2004)*

Expression in OV10 cells of either wild-type CD47 or its extracellular IgV domain linked to a glycosylphosphatidylinositol anchor-(IgV-GPI) enhanced ligand-induced $\alpha_v\beta_3$ activation as detected by the binding of LIBS1 and LIBS6 mAbs. The amplitude of LIBS binding was greater with both CD47 and IgV-GPI expression, indicating an increase in the population of "activable" integrin molecules. Expression of either CD47 species also increased $\alpha_v\beta_3$-mediated adhesion to vitronectin, and to surfaces coated with the anti-β_3 antibody AP3, because of enhanced clustering of $\alpha_v\beta_3$ as confirmed by chemical cross-linking. Cholesterol depletion with methyl-β-cyclodextrin did not prevent the increase in anti-LIBS binding, but reduced cell adhesion to vitronectin and AP3. However, cells expressing CD47 were partially insulated against this disruption, and IgV-GPI was even more effective. Both CD47 and IgV-GPI were found in cholesterol-rich rafts prepared in the absence of detergent, but only CD47 could recruit $\alpha_v\beta_3$ and its associated signaling molecules to these domains. Thus CD47-$\alpha_v\beta_3$ complexes in cholesterol-rich raft domains appear to engage in G_i-dependent signaling whereas CD47-$\alpha_v\beta_3$ interactions that lead to integrin clustering are also detergent resistant, but are insensitive to cholesterol depletion and do not require the transmembrane region of CD47.

3.495 Brain-specific deletion of neuropathy target esterase/*swisscheese* results in neurodegeneration
Akassoglou, K. et al
*PNAS, **101(14)**, 5075-5080 (2004)*

Neuropathy target esterase (NTE) is a neuronal membrane protein originally identified for its property to be modified by organo-phosphates (OPs), which in humans cause neuropathy characterized by axonal degeneration. *Drosophila* mutants for the homolog gene of *NTE, swisscheese* (*sws*), indicated a possible involvement of *sws* in the regulation of axon-glial cell interaction during glial wrapping. However, the role of NTE/*sws* in mammalian brain pathophysiology remains unknown. To investigate NTE function *in vivo*, we used the cre/loxP site-specific recombination strategy to generate mice with a specific deletion of NTE in neuronal tissues. Here we show that loss of NTE leads to prominent neuronal pathology in the hippocampus and thalamus and also defects in the cerebellum. Absence of NTE resulted in disruption of the endoplasmic reticulum, vacuolation of nerve cell bodies, and abnormal reticular aggregates. Thus, these results identify a physiological role for NTE in the nervous system and indicate that a loss-of-function mechanism may contribute to neurodegenerative diseases characterized by vacuolation and neuronal loss.

3.496 The G protein-coupled receptor rhodosin in the native membrane
Fotiadis, D. et al
*FEBS Lett., **564**, 281-288 (2004)*

The higher-order structure of G protein-coupled receptors (GPCRs) in membranes may involve dimerization and formation of even larger oligomeric complexes. Here, we have investigated the organization of the prototypical GPCR rhodopsin in its native membrane by electron and atomic force microscopy (AFM). Disc membranes from mice were isolated and observed by AFM at room temperature. In all experimental conditions, rhodopsin forms structural dimers organized in paracrystalline arrays. A semi-empirical molecular model for the rhodopsin paracrystal is presented validating our previously reported results. Finally, we compare our model with other currently available models describing the supramolecular structure of GPCRs in the membrane.

3.497 Copatching and lipid raft association of different viral glycoproteins expressed on the surfaces of Pseudorabies virus-infected cells
Favoreel, H.W., Mettenleiter, T.C. and Nauwynck, H.J.
*J. Virol., **78(10)**, 5279-5287 (2004)*

Pseudorabies virus (PRV) is a swine alphaherpesvirus that is closely related to human herpes simplex virus (HSV). Both PRV and HSV express a variety of viral envelope glycoproteins in the plasma membranes of infected cells. Here we show that at least four major PRV glycoproteins (gB, gC, gD, and gE) in the plasma membrane of infected swine kidney cells and monocytes seem to be linked, since monospecific antibody-induced patching of any one of these proteins results in copatching of the others. Further, for all four PRV glycoproteins, monospecific antibody-induced patches were enriched in GM1, a typical marker of lipid raft microdomains, but were excluded for transferrin receptor, a nonraft marker, suggesting that these viral proteins may associate with lipid rafts. However, only gB and, to a lesser extent, gE were found in lipid raft fractions by using detergent floatation assays, indicating that gC and gD do not show strong lipid raft association. Addition of methyl-ß-cyclodextrin (MCD), a cholesterol-depleting agent that is commonly used to disrupt lipid rafts, only slightly reduced copatching efficiency between the different viral proteins, indicating that other factors, perhaps tegument-glycoprotein interactions, may be important for the observed copatching events. On the other hand, MCD strongly reduced polarization of the antibody-induced viral glycoprotein patches to a cap structure, a gE-dependent process that has been described for specific PRV- and HSV-infected cells. Therefore, we hypothesize that efficient gE-mediated capping of antibody-antigen patches may require the lipid raft-associated signal transduction machinery.

3.498 Endogenous SHIP2 does not localize in lipid rafts in 3T3-L1 adipocytes
Jacobs, C., Onnockx, S., Vandenbroere, I. and Pirson, I.
*FEBS Lett., **565**, 70-74 (2004)*

SH2 domain containing inositol polyphosphate 5-phosphatase (SHIP2) dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) into phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2). SHIP2 knock-out mice demonstrated that SHIP2 acts as a negative regulator of insulin cascade in vivo. Our two-hybrid study showed that SHIP2 interacts with c-Cbl associated protein (CAP) and c-Cbl, implicated in the insulin signaling. As some proteins implicated in insulin signaling, like insulin receptor, CAP, c-Cbl or TC10, were reported to localize in lipid rafts, we addressed the same question for SHIP2. SHIP2 was detected in the non-raft fraction in CHO-IR, C2C12 myotubes and 3T3-L1 adipocytes except when it is overexpressed in CHO-IR, where we detected SHIP2 in the raft fraction.

3.499 Uptake and transsport of high-density lipoprotein (HDL) and HDL-associated α-tocopherol by an *in vitro* blood-brain barrier model
Balazs, Z. et al
J. Neurochem., ***89***, *939-950 (2004)*

The present study aimed to investigate pathways that contribute to uptake and transcytosis of high-density lipoproteins (HDLs) and HDL-associated α-tocopherol (αTocH) across an *in vitro* model of the blood-brain barrier (BBB). In primary porcine brain capillary endothelial cells HDL-associated αTocH was taken up in 10-fold excess of HDL holoparticles, indicating efficient selective uptake, a pathway mediated by scavenger receptor class B, type I (SR-BI). SR-BI was present in caveolae of brain capillary endothelial cells and expressed almost exclusively at the apical membrane. Disruption of caveolae with methyl-β-cyclodextrin (CDX) resulted in (mis)sorting of SR-BI to the basolateral membrane. Immunohistochemistry of porcine brain cryosections revealed SR-BI expression on brain capillary endothelial cells and presumably astrocytic endfeet. HDL-associated [^{14}C]αTocH taken up by brain capillary endothelial cells was recovered in sucrose gradient fractions containing the majority of cellular caveolin-1, the major caveolae-associated protein. During mass transfer studies using αTocH-enriched HDL, approximately 50% of cellular αTocH was recovered with the bulk of cellular caveolin-1 and SR-BI. Efflux experiments revealed that a substantial amount of cell-associated [^{14}C]αTocH could be mobilized into the culture medium. In addition, apical-to-basolateral transport of HDL holoparticles and HDL-associated αTocH was saturable. Results from the present study suggest that part of cerebral apolipoprotein A-I and αTocH originates from plasma HDL transcytosed across the BBB and that caveolae-located SR-BI facilitates selective uptake of HDL-associated αTocH at the BBB.

3.500 Caveolar and lipid raft localization of the growth hormone receptor ans its signaling elements
Yang, N., Huang, Y., Jiang, J. And Frank, S.J.
J. Biol. Chem., ***279(20)***

The growth hormone receptor (GHR) is a cell surface receptor that mediates the somatogenic and metabolic effects of the growth hormone (GH). GHR signaling is transduced via the receptor-associated cytoplasmic tyrosine kinase called Janus protein kinase 2 (JAK2). The major intracellular signaling systems activated by JAK2 in response to GH include the signal transducer and activator of transcription (STAT) 5 and extracellular signal-regulated kinase (ERK)-1 and -2 pathways. In this report, we investigate the role of cholesterol-rich plasma membrane microdomains (caveolae and lipid rafts) in GH signaling. By subcellular fractionation of the GH-responsive 3T3-F442A murine preadipocyte, we found dramatic enrichment (6.7-fold) of plasma membrane GHR in the caveolae membranes (CM). JAK2 was also represented in the CM fraction, but was less enriched (2.5-fold) than GHR. ERK1/2 and the important ERK pathway upstream small adaptor protein, Grb2 (growth factor receptor-bound protein 2), were also enriched in caveolae (2.3- and 8.3-fold, respectively), but STAT5 was barely detected in the same fraction. Correspondingly, GH-induced tyrosine-phosphorylated GHR, JAK2, and ERK1/2 were highly represented in the CM fraction, whereas tyrosine-phosphorylated STAT5 was enriched in the non-membranous fraction of the post-nuclear supernatant. Additionally, GH induced further accumulation of GHR, Grb2, and SHC proteins in the CM fraction. Interestingly, treatment of the cells with the caveolae-disrupting agent, methyl-β-cyclodextrin (mβCD), selectively inhibited GH-induced ERK1/2 activation but not STAT5 phosphorylation; repletion of cholesterol in mβCD-treated cells restored GH-induced ERK activation. Comparison of 3T3-F442A cells with the GHR-expressing human IM-9 lymphoblasts revealed similar enrichment of GHR in the lipid raft fraction of IM-9 as in the CM fraction of 3T3-F442A, but there were dramatic differences in the ERKs and Grb2. The IM-9 cell, in which ERKs are not activated by GH, displayed no enrichment of ERKs and Grb2 in the lipid raft fraction. Our results suggest that localization of GHRs in the CM fraction of the plasma membrane plays important roles in signaling.

3.501 A role for myosin-1A in the localization of a brush border disaccharidase
Tyska, M.J. and Mooseker, M.S.
J. Cell Biol., ***165(3)***, *395-405 (2004)*

To gain insight regarding myosin-1A (M1A) function, we expressed a dominant negative fragment of this motor in the intestinal epithelial cell line, CACO-2_{BBE}. Sucrase isomaltase (SI), a transmembrane disaccharidase found in microvillar lipid rafts, was missing from the brush border (BB) in cells expressing

this fragment. Density gradient centrifugation, affinity purification, and immunopurification of detergent-resistant membranes isolated from CACO-2_{BBE} cells and rat microvilli (MV) all indicate that M1A and SI reside on the same population of low density (~1.12 g/ml) membranes. Chemical cross-linking of detergent-resistant membranes from rat MV indicates that SI and M1A may interact in a lipid raft complex. The functional significance of such a complex is highlighted by expression of the cytoplasmic domain of SI, which results in lower levels of M1A and a loss of SI from the BB. Together, these studies are the first to assign a specific role to M1A and suggest that this motor is involved in the retention of SI within the BB.

3.502 Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors
Dove, S.K. et al
*EMBO J., **23**, 1922-1933 (2004)*

Phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2), made by Fab1p, is essential for vesicle recycling from vacuole/lysosomal compartments and for protein sorting into multivesicular bodies. To isolate PtdIns(3,5)P_2 effectors, we identified *Saccharomyces cerevisiae* mutants that display *fab1*Δ-like vacuole enlargement, one of which lacked the *SVP1/*YFR021w/*ATG18* gene. Expressed Svp1p displays PtdIns(3,5)P_2 binding of exquisite specificity, GFP-Svp1p localises to the vacuole membrane in a Fab1p-dependent manner, and *svp1*Δ cells fail to recycle a marker protein from the vacuole to the Golgi. Cells lacking Svp1p accumulate abnormally large amounts of PtdIns(3,5)P_2. These observations identify Svp1p as a PtdIns(3,5)P_2 effector required for PtdIns(3,5)P_2-dependent membrane recycling from the vacuole. Other Svp1p-related proteins, including human and *Drosophila* homologues, bind PtdIns(3,5)P_2 similarly. Svp1p and related proteins almost certainly fold as ß-propellers, and the PtdIns(3,5)P_2-binding site is on the ß-propeller. It is likely that many of the Svp1p-related proteins that are ubiquitous throughout the eukaryotes are PtdIns(3,5)P_2 effectors. Svp1p is not involved in the contributions of *FAB1*/PtdIns(3,5)P_2 to MVB sorting or to vacuole acidification and so additional PtdIns(3,5)P_2 effectors must exist.

3.503 Differential regulation of cytosolic and peroximal bile acid amidation by PPARα activation favors the formation of unconjugated bile acids
Solaas, K. et al
*J. Lipid. Res.,**45**, 1051-1060 (2004)*

In human liver, unconjugated bile acids can be formed by the action of bile acid-CoA thioesterases (BACTEs), whereas bile acid conjugation with taurine or glycine (amidation) is catalyzed by bile acid-CoA:amino acid *N*-acyltransferases (BACATs). Both pathways exist in peroxisomes and cytosol. Bile acid amidation facilitates biliary excretion, whereas the accumulation of unconjugated bile acids may become hepatotoxic. We hypothesized that the formation of unconjugated and conjugated bile acids from their common substrate bile acid-CoA thioesters by BACTE and BACAT is regulated via the peroxisome proliferator-activated receptor α(PPARα). Livers from wild-type and PPARα-null mice either untreated or treated with the PPARα activator WY-14,643 were analyzed for BACTE and BACAT expression. The total liver capacity of taurochenodeoxycholate and taurocholate formation was decreased in WY-14,643-treated wild-type mice by 60% and 40%, respectively, but not in PPARα-null mice. Suppression of the peroxisomal BACAT activity was responsible for the decrease in liver capacity, whereas cytosolic BACAT activity was essentially unchanged by the treatment. In both cytosol and peroxisomes, the BACTE activities and protein levels were upregulated 5- to 10-fold by the treatment. These effects caused by WY-14,643 treatment were abolished in PPARα-null mice.
The results from this study suggest that an increased formation of unconjugated bile acids occurs during PPARα activation.

3.504 Passive and active inclusion of host proteins in human immunodeficiency virus type 1 Gag particles during budding at the plasma membrane
Hammerstedt, M. and Garoff, H.
*J. Virol., **78(11)**, 5686-5697 (2004)*

Human immunodeficiency virus type 1 particles form by budding at the surface of most cell types. In this process, a piece of the plasma membrane is modified into an enveloped virus particle. The process is driven by the internal viral protein $Pr55^{gag}$. We have studied how host proteins in the membrane are dealt with by $Pr55^{gag}$ during budding. Are they included in or excluded from the particle? The question was approached by measuring the relative concentrations of host and viral proteins in the envelope of $Pr55^{gag}$ particles and in their donor membranes in the cell. We observed that the bulk of the host proteins, including actin and clathrin, were passively included into the virus-like Gag particles. This result suggests that budding by

$Pr55^{gag}$ proceeds without significant alteration of the original host protein composition at the cell membrane. Nevertheless, some proteins were concentrated in the particles, and a few were excluded. The concentrated proteins included cyclophilin A and Tsg-101. These were recruited to the plasma membrane by $Pr55^{gag}$. The membrane-bound cyclophilin A was concentrated into particles as efficiently as $Pr55^{gag}$, whereas Tsg-101 was concentrated more efficiently. The latter finding is consistent with a role for Tsg-101 in Gag particle release.

3.505 Functions of pancreatic β cells and adipocytes in bombesin receptor subtype-3-deficient mice
Nakamichi, Y. et al
Biochem. Biophys. Res. Comm., ***318***, *698-703 (2004)*

We previously reported that mice lacking bombesin receptor subtype-3 (BRS-3) exhibit mild late-onset obesity and glucose intolerance [Nature 390 (1997) 160]. To examine the mechanism by which glucose intolerance is developed in these mice, we studied insulin release and proinsulin biosynthesis in isolated pancreatic islets and glucose uptake and facilitative glucose transporter (GLUT)-4 translocation in adipose tissues. Although islet insulin contents and the size and number of islets of Langerhans in BRS-3-deficient mice decreased, there was no difference in glucose-stimulated insulin release and proinsulin biosynthesis between BRS-3-deficient and wild-type control mice. In contrast, adipose tissues exhibited a marked difference: the uptake of [^{14}C]2-deoxy-D-glucose by adipocytes isolated from BRS-3-deficient mice was not stimulated by 10^{-7} M insulin addition, and membrane fractionation analysis showed that GLUT4 was barely detected in the fraction of plasma membrane in BRS-3-deficient mice in the presence of 10^{-7} M insulin. Quantitative reverse transcription-PCR (RT-PCR) showed that mRNA levels of GLUT4, insulin receptor, insulin receptor substrate (IRS)-1 and IRS-2, syntaxin 4, SNAP23, and VAMP-2 in adipose tissues of BRS-3-deficient mice were unchanged compared with those in wild-type control mice. We concluded that impaired glucose metabolism observed in BRS-3-deficient mice was mainly caused by impaired GLUT4 translocation in adipocytes.

3.506 Analysis of lipid rafts in T cell
Thomas, S., Preda-Pais, A., Casares, S. and Brumeanu, T-D.
Mol. Immunol., ***41***, *399-409 (2004)*

The plasma membrane of T cells is made of a combination of glycosphingolipids and protein receptors organized in glycolipoprotein microdomains termed lipid rafts. The structural assembly of lipid rafts was investigated by various physical and biochemical assays. Depending on the differentiation status of T cells, the lipid rafts seclude various protein receptors involved in T cell signaling, cytoskeleton reorganization, membrane trafficking, and the entry of infectious organisms into the cells. This review article summarizes the most common methods, and their limits and advantages for analyzing the composition and assembly of lipid rafts with protein receptors into lipid rafts microdomains in plasma membrane of T cells. It also includes new methods such as ELISA/Polysorp and flow cytometry, and a combined sucrose gradient centrifugation–FPLC–Western blot strategy developed in our laboratory to study non-covalent interactions between the GM1 glycosphingolipid and protein receptors in plasma membrane of T cells.

3.507 Functional domains in presenilin. The TYR-288 residue controls γ-secretase activity and endoprotoeolysis
Laudon, H. et al
J. Biol. Chem., ***279(23)***, *23925-23932 (2004)*

Processing of the Alzheimer amyloid precursor protein (APP) into the amyloid β-protein and the APP intracellular domain is a proteolysis event mediated by the γ-secretase complex where presenilin (PS) proteins are key constituents. PS is subjected to an endoproteolytic cleavage, generating a stable heterodimer composed of an N-terminal and a C-terminal fragment. Here we aimed at further understanding the role of PS in endoproteolysis, in proteolytic processing of APP and Notch, and in assembly of the γ-secretase complex. By using a truncation protocol and alanine scanning, we identified Tyr-288 in the PS1 N-terminal fragment as critical for PS-dependent intramembrane proteolysis. Further mutagenesis of the 288 site identified mutants differentially affecting endoproteolysis and γ-secretase activity. The Y288F mutant was endoproteolyzed to the same extent as wild type PS but increased the amyloid β-protein 42/40 ratio by ~75%. In contrast, the Y288N mutant was also endoproteolytically processed but was inactive in reconstituting γ-secretase in PS null cells. The Y288D mutant was deficient in both endoproteolysis and γ-secretase activity. All three mutant PS1 molecules were incorporated into γ-secretase complexes and stabilized Pen-2 in PS null cells. Thus, mutations at Tyr-288 do not affect γ-secretase complex assembly but can differentially control endoproteolysis and γ-secretase activity.

3.508 The T cell receptor γ chain alternate reading frame protein (TARP), a prostate-specific protein localized in mitochondria

Maeda, H. et al

J. Biol. Chem., ***279(23)***, *24561-24568 (2004)*

We previously showed that mRNA encoding TARP (T cell receptor γ chain alternate reading frame protein) is exclusively expressed in the prostate in males and is up-regulated by androgen in LNCaP cells, an androgen-sensitive prostate cancer cell line. We have now developed an anti-TARP monoclonal antibody named TP1, and show that TARP protein is up-regulated by androgen in both LNCaP and MDA-PCa-2b cells. We used TP1 to determine the subcellular localization of TARP by Western blotting following subcellular fractionation and immunocytochemistry. Both methods showed that TARP is localized in the mitochondria of LNCaP cells, MDA-PCa-2b cells, and PC-3 cells transfected with a TARP-expressing plasmid. We also transfected a plasmid encoding TARP fused to green fluorescent protein into LNCaP, MDA-Pca-2b, and PC-3 cells and confirmed its specific mitochondrial localization in living cells. Fractionation of mitochondria shows that TARP is located in the outer mitochondrial membrane. Immunohistochemistry using a human prostate cancer sample showed that TP1 reacted in a dot-like cytoplasmic pattern consistent with the presence of TARP in mitochondria. These data demonstrate that TARP is the first prostate-specific protein localizing in mitochondria and indicate that TARP, an androgen-regulated protein, may act on mitochondria to carry out its biological functions.

3.509 Lipid raft polarization contributes to hyphal growth in *Candida albicans*

Martin, S.W. and Konopka, J.B.

*Eukaryotic cell, **3(3)**, 675-684 (2004)*

The polarization of sterol- and sphingolipid-enriched domains (lipid rafts) has been linked to morphogenesis and cell movement in diverse cell types. In the yeast *Saccharomyces cerevisiae*, a dramatic polarization of sterol-rich domains to the shmoo tip was observed in pheromone-induced cells (M. Bagnat and K. Simons, Proc. Natl. Acad. Sci. USA **99:**14183-14188, 2002). We therefore examined whether plasma membrane lipid polarization contributes to the ability of the fungal pathogen *Candida albicans* to grow in a highly polarized manner to form hyphae. Interestingly, staining with filipin revealed that membrane sterols were highly polarized to the leading edge of growth during all stages of hyphal growth. Budding and pseudohyphal cells did not display polarized staining. Filipin staining was also enriched at septation sites in hyphae, where colocalization with septin proteins was observed, suggesting a role for the septins in forming a boundary domain. Actin appeared to play a role in sterol polarization and hyphal morphogenesis in that both were disrupted by low concentrations of latrunculin A that did not prevent budding. Furthermore, blocking either sphingolipid biosynthesis with myriocin or sterol biosynthesis with ketoconazole resulted in a loss of ergosterol polarization and caused abnormal hyphal morphogenesis, suggesting that lipid rafts are involved. Since hyphal growth is required for the full virulence of *C. albicans*, these results suggest that membrane polarization may contribute to the pathogenesis of this organism.

3.510 CD44 interaction with Na^+-H^+ exchanger (NHE1) creates acidic microenvironment leading to hyaluronidase-2 and cathepsin B activation and breast tumor cell invasion

Bourguignon, L.Y.W., Singleton, P.A., Diedrich, F., Stern, R. and Gilad, E.

J. Biol. Chem., ***279(26)***, *26691-27007 (2004)*

We have explored CD44 (a hyaluronan (HA) receptor) interaction with a Na^+-H^+ exchanger (NHE1) and hyaluronidase-2 (Hyal-2) during HA-induced cellular signaling in human breast tumor cells (MDA-MB-231 cell line). Immunological analyses demonstrate that CD44s (standard form) and two signaling molecules (NHE1 and Hyal-2) are closely associated in a complex in MDA-MB-231 cells. These three proteins are also significantly enriched in cholesterol and ganglioside-containing lipid rafts, characterized as caveolin and flotillin-rich plasma membrane microdomains. The binding of HA to CD44 activates Na^+-H^+ exchange activity which, in turn, promotes intracellular acidification and creates an acidic extracellular matrix environment. This leads to Hyal-2-mediated HA catabolism, HA modification, and cysteine proteinase (cathepsin B) activation resulting in breast tumor cell invasion. In addition, we have observed the following: (i) HA/CD44-activated Rho kinase (ROK) mediates NHE1 phosphorylation and activity, and (ii) inhibition of ROK or NHE1 activity (by treating cells with a ROK inhibitor, Y27632, or NHE1 blocker, *S*-(*N*-ethyl-*N*-isopropyl) amiloride, respectively) blocks NHE1 phosphorylation/Na^+-H^+ exchange activity, reduces intracellular acidification, eliminates the acidic environment in the extracellular matrix, and suppresses breast tumor-specific behaviors (*e.g.* Hyal-2-mediated HA modification, cathepsin B

activation, and tumor cell invasion). Finally, down-regulation of CD44 or Hyal-2 expression (by treating cells with CD44 or Hyal-2-specific small interfering RNAs) not only inhibits HA-mediated CD44 signaling (*e.g.* ROK-mediated Na^+-H^+ exchanger reaction and cellular pH changes) but also impairs oncogenic events (*e.g.* Hyal-2 activity, hyaluronan modification, cathepsin B activation, and tumor cell invasion). Taken together, our results suggest that CD44 interaction with a ROK-activated NHE1 (a Na^+-H^+ exchanger) in cholesterol/ganglioside-containing lipid rafts plays a pivotal role in promoting intracellular/extracellular acidification required for Hyal-2 and cysteine proteinase-mediated matrix degradation and breast cancer progression.

3.511 Carboxyl ester lipase cofractionates with scavenger receptor BI in hepatocyte lipid rafts and enhances selective uptake and hydrolysis of cholesteryl esters from HDL_3
Camarota, L.M., Chapman, J.M., Hui, D.Y. and Howles, P.N.
J. Biol. Chem., ***279(26)***, *27599-27606 (2004)*

Cholesteryl esters are selectively removed from high density lipoproteins by hepatocytes and steroidogenic cells through a process mediated by scavenger receptor BI. In the liver this cholesterol is secreted into bile, primarily as free cholesterol. Previous work showed that carboxyl ester lipase enhanced selective uptake of cholesteryl ether from high density lipoprotein by an unknown mechanism. Experiments were performed to determine whether carboxyl ester lipase plays a role in scavenger receptor BI-mediated selective uptake. When added to cultures of HepG2 cells, carboxyl ester lipase cofractionated with scavenger receptor BI and [^{3}H]cholesteryl ether-labeled high density lipoprotein in lipid raft fractions of cell homogenates. Confocal microscopy of immunostained carboxyl ester lipase and scavenger receptor BI showed a close association of these proteins in HepG2 cells. The enzyme and receptor also cofractionated from homogenates of mouse liver using two different fractionation methods. Antibodies that block scavenger receptor BI function prevented carboxyl ester lipase stimulation of selective uptake in primary hepatocytes from carboxyl ester lipase knockout mice. Heparin blockage of cell-surface proteoglycans also prevented carboxyl ester lipase stimulation of cholesteryl ester uptake by HepG2 cells. Inhibition of carboxyl ester lipase activity in HepG2 cells reduced hydrolysis of high density lipoprotein-cholesteryl esters ~40%. *In vivo*, hydrolysis was similarly reduced in lipid rafts from the livers of carboxyl ester lipase-null mice compared with control animals. Primary hepatocytes from these mice yielded similar results. The data suggest that carboxyl ester lipase plays a physiological role in hepatic selective uptake and metabolism of high density lipoprotein cholesteryl esters by direct and indirect interactions with the scavenger receptor BI pathway.

3.512 PSPN/GFRα4 has a significantly weaker capacity thab GDNF/GFRα1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth
Yang, J. et al
FEBS Lett., ***569***, *267-271 (2004)*

Previously, it was shown that the recruitment of RET into lipid rafts by glial cell line-derived neurotrophic factor (GDNF)/GFRα1 is crucial for efficient signal transduction. Here, we show that the mouse GFRα4 is a functional, *N*-glycosylated, glycosylphosphatidylinositol (GPI)-anchored protein, which mediates persephin (PSPN)-induced phosphorylation of RET, but has an almost undetectable capacity to recruit RET into the 0.1% Triton X-100 insoluble membrane fraction. In spite of this, PSPN/mGFRα4 promotes neurite outgrowth in PC6-3 cells and survival of cerebellar granule neurons. As we show that also human PSPN/GFRα4 is unable to recruit RET into lipid rafts, we propose that the mammalian GFRα4 in this respect differs from GFRα1.

3.513 Sphingolipid C4 hydroxylation influences properties of yeast detergent-insoluble glycolipid-enriched membranes
Idkowiak-Baldys, J., Grilley, M.M. and Takemoto, J.Y.
FEBS Lett., ***569***, *272-276 (2004)*

Sphingoid base C4 hydroxylation is required for syringomycin E action on the yeast plasma membrane. Detergent-insoluble glycolipid-enriched membranes (DIGs) from a yeast strain lacking C4 hydroxylated sphingoid bases (*sur2*Δ) are composed of linear membrane fragments instead of vesicular structures observed for wild-type DIGs, though they have similar lipid compositions and amounts of DIG marker proteins. Light-scattering bands collected from *sur2*Δ after centrifugation of Triton X-100-treated cell lysates in continuous density gradients have lower buoyant densities than that of the wild-type. The results

show that C4 hydroxylation influences the physical and structural properties of DIGs and suggest that syringomycin E interacts with lipid rafts.

3.514 Membrane order conservation in raft and non-raft regions of hepatocyte plasma membranes from thermally acclimated rainbow trout
Zehmer, J.K. and Hazel, J.R.
*Biochim, Biophys. Acta., **1664**, 108-116 (2004)*

Homeoviscous adaptation (HVA), the thermal conservation of membrane fluidity/order at different body temperatures, has been observed to varying degrees in different membranes. However, HVA has not been studied in raft and non-raft regions of the plasma membrane (PM) separately. Rafts are ordered PM microdomains implicated in signal transduction, membrane traffic and cholesterol homeostasis. Using infrared spectroscopy, we measured order in raft-enriched PM (raft) and raft-depleted PM (RDPM) isolated from hepatocytes of rainbow trout (*Oncorhynchus mykiss*) acclimated to 5 and 20 °C. We found approximately 130% and 90% order compensation in raft and RDPM, respectively, suggesting their independent regulation. Raft was more ordered than RDPM in the warm-acclimated trout, a difference fully explained by a 58% enrichment of cholesterol, compared to RPDM. Unexpectedly, raft and RDPM from cold-acclimated trout did not differ in cholesterol content or order. Freezing the membrane samples during preparation had no effect on order. Treatment with cyclodextrin depleted cholesterol by 36%, 56%, and 55%, producing significant decreases in order in raft and RDPM from warm-acclimated trout and RDPM from cold-acclimated trout, respectively. However, a 69% depletion of cholesterol from raft from cold-acclimated trout had no significant effect on order. This result, and the lack of a difference in order between raft and RDPM, suggests that raft and non-raft PM in cold-acclimated trout are not spatially segregated by phase separation due to cholesterol.

3.515 Cellular distribution of lysyl-tRNA synthetase and its interaction with Gag during human immunodeficiency virus type 2 assembly
Halwani, R. et al
*J. Virol., **78(14)**, 7553-7564 (2004)*

Lysyl-tRNA synthetase (LysRS) is packaged into human immunodeficiency virus type 1 (HIV-1) via its interaction with Gag, and this enzyme facilitates the selective packaging of $tRNA_3^{Lys}$, the primer for initiating reverse transcription, into HIV-1. The Gag/LysRS interaction is detected at detergent-resistant membrane but not in membrane-free cell compartments that contain Gag and LysRS. LysRS is found (i) in the nucleus, (ii) in a cytoplasmic high-molecular-weight aminoacyl-tRNA synthetase complex (HMW aaRS complex), (iii) in mitochondria, and (iv) associated with plasma membrane. The cytoplasmic form of LysRS lacking the mitochondrial import signal was previously shown to be efficiently packaged into virions, and in this report we also show that LysRS compartments in nuclei, in the HMW aaRS complex, and at the membrane are also not required as a primary source for viral LysRS. Exogenous mutant LysRS species unable to either enter the nucleus or bind to the cell membrane are still incorporated into virions. Many HMW aaRS components are not packaged into the virion along with LysRS, and the interaction of LysRS with p38, a protein that binds tightly to LysRS in the HMW aaRS complex, is not required for the incorporation of LysRS into virions. These data indicate that newly synthesized LysRS may interact rapidly with Gag before the enzyme has the opportunity to move to the above-mentioned cellular compartments. In confirmation of this idea, we found that newly synthesized LysRS is associated with Gag after a 10-min pulse with [^{35}S]cysteine/methionine. This observation is also supported by previous work indicating that the incorporation of LysRS into HIV-1 is very sensitive to the inhibition of new synthesis of LysRS.

3.516 Microtubules regulate angiotensin II type 1 receptor and Rac1 localization in caveolae/lipid rafts
Zuo, L., Ushio-Fukai, M., Hilenski, L.L. and Alexander, R.W.
*Arterioscler. Thromb. Vasc. Biol., **24**, 1223-1228 (2004)*

Objective— Microtubules are important in signal transduction temporal–spatial organization. Full expression of angiotensin II (Ang II) signaling in vascular smooth muscle cells (VSMCs) is dependent on the reactive oxygen species (ROS) derived from nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase and the dynamic association of the Ang II type 1 receptor (AT_1R) with caveolae/lipid rafts. Translocation of the small GTPase Rac1 to the plasma membrane is an essential step for activation of NAD(P)H oxidase; however, its precise localization in the plasma membrane after agonist stimulation and

how it is targeted are unknown. We hypothesized that microtubules are involved in regulating multiphasic Ang II signaling events in VSMC.
Methods and Results— We show that Ang II promotes Rac1 and AT_1R trafficking into caveolae/lipid rafts, which is blocked by disruption of microtubules with nocodazole. As a consequence, nocodazole significantly inhibits Ang II–stimulated H_2O_2 production, its downstream ROS-dependent epidermal growth factor receptor transactivation, Akt phosphorylation, and vascular hypertrophy without affecting Rac1 activation or ROS-independent extracellular signal-regulated kinase 1/2 phosphorylation.
Conclusions— These results suggest that proper Rac1 and AT_1R trafficking into caveolae/lipid rafts requires the integrity of microtubules and provide insight into the essential role of microtubules for the spatial–temporal organization of ROS-dependent and caveolae/lipid rafts–dependent AT_1R signaling linked to vascular hypertrophy.
The role of microtubules in angiotensin II (Ang II) signaling remains unknown. We demonstrate that Ang II promotes Rac1 and Ang II type 1 receptor trafficking into the caveolae/lipid rafts, which requires the integrity of microtubules. We also found that intact microtubules mediate Ang II–stimulated H_2O_2 production, its downstream EGF-R transactivation, Akt phosphorylation, and vascular hypertrophy.

3.517 Stoichiometry of the T-cell receptor-CD3 complex and key intermediates assembled in the endoplasmic reticulum
Call, M.E., Pyrdol, J. and Wucherpfennig, K.W.
*EMBO J., **22**, 2348-2357 (2004)*

The T-cell receptor (TCR)–CD3 complex is critical for T-cell development and function, and represents one of the most complex transmembrane receptors. Models of different stoichiometry and valency have been proposed based on cellular experiments and these have important implications for the mechanisms of receptor triggering. Since determination of receptor stoichiometry in T-cells is not possible due to the presence of previously synthesized, unlabeled receptor components with different half-lives, we examined the stoichiometry of the receptor assembled in endoplasmic reticulum (ER) microsomes of B-cell origin. The stoichiometric relationship among all subunits was directly determined using intact radiolabeled TCR–CD3 complexes that were isolated with a sequential, non-denaturing immunoprecipitation method, and identical results were obtained with two detergents belonging to different structural classes. The results firmly establish that the αß TCR–CD3 complex assembled in the ER is monovalent and composed of one copy of the TCRαß, CD3δε, CD3γε and ζ–ζ dimers.

3.518 PKD1/PKCμ promotes αvβ3 integrin recycling and delivery to nascent focal adhesions
Woods, A.J., White, D.P., Caswell, P.T. and Norman, J.C.
*EMBO J., **23**, 2531-2543 (2004)*

To identify kinases that regulate integrin recycling, we have immunoprecipitated αvß3 integrin from NIH 3T3 fibroblasts in the presence and absence of primaquine (a drug that inhibits receptor recycling and leads to accumulation of integrins in endosomes) and screened for co-precipitating kinases. Primaquine strongly promoted association of αvß3 integrin with PKD1, and fluorescence microscopy indicated that integrin and PKD1 associate at a vesicular compartment that is downstream of a Rab4-dependent transport step. PKD1 association was mediated by the C-terminal region of the ß3 integrin cytodomain, and mutants of ß3 that were unable to recruit PKD1 did not recycle in a PDGF-dependent fashion. Furthermore, suppression of endogenous PKD1 levels by RNAi, or overexpression of catalytically inactive PKD1 inhibited PDGF-dependent recycling of αvß3 from early endosomes to the plasma membrane and blocked recruitment of α vß3 to newly formed focal adhesions during cell spreading. These data indicate that PKD1 influences cell migration by directing vesicular transport of the αvß3 integrin heterodimer.

3.519 Human Doppel and prion protein share common membrane microdomains and internalization pathways
Massimino, M.L. et al
*Int. J. Biochem. and Cell Biol., **36**, 2016-2031 (2004)*

Doppel is the first identified homologue of the prion protein (PrP^c) implicated in prion disease. Doppel is considered an N-truncated form of PrP^c, and shares with PrP^c several structural and biochemical features. When over expressed in the brain of some PrP knockout animals, it provokes cerebellar ataxia. As this phenotype is rescued by reintroducing the PrP gene, it has been suggested that Doppel and PrP^c have antagonistic functions and may compete for a common ligand. However, a direct interaction between the two proteins has recently been observed.

To investigate whether the neuronal environment is suitable for such possibility, human Doppel and PrP^c were expressed separately, or together, in neuroblastoma cells, and then studied by biochemical and immunomicroscopic tools, as well as in intact cells expressing fluorescent fusion constructs.
The results demonstrate that Doppel and PrP^c co-patch extensively at the plasma membrane, and get internalized together after ganglioside cross-linking by cholera toxin or addition of an antibody against only one of the proteins. These processes no longer occur if the integrity of rafts is disrupted. We also show that, whereas each protein expressed alone occupies Triton X-100-insoluble membrane microdomains, co-transfected Doppel and PrP^c redistribute together into a less ordered lipidic environment. All these features are consistent with interactions occurring between Doppel and PrP^c in our neuronal cell model.

3.520 Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment
Adolfsen, B., Sarawati, S., Yoshihara, M. and Littleton, J.T.
J. Cell Biol., ***166(2)***, *249-260 (2004)*

The synaptotagmin family has been implicated in calcium-dependent neurotransmitter release, although Synaptotagmin 1 is the only isoform demonstrated to control synaptic vesicle fusion. Here, we report the characterization of the six remaining synaptotagmin isoforms encoded in the *Drosophila* genome, including homologues of mammalian Synaptotagmins 4, 7, 12, and 14. Like Synaptotagmin 1, Synaptotagmin 4 is ubiquitously present at synapses, but localizes to the postsynaptic compartment. The remaining isoforms were not found at synapses (Synaptotagmin 7), expressed at very low levels (Synaptotagmins 12 and 14), or in subsets of putative neurosecretory cells (Synaptotagmins α and ß). Consistent with their distinct localizations, overexpression of Synaptotagmin 4 or 7 cannot functionally substitute for the loss of Synaptotagmin 1 in synaptic transmission. Our results indicate that synaptotagmins are differentially distributed to unique subcellular compartments. In addition, the identification of a postsynaptic synaptotagmin suggests calcium-dependent membrane-trafficking functions on both sides of the synapse.

3.521 *Drosophila* Wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine
Zhai, L., Chaturvedi, D. and Cumberledge, S.
J. Biol. Chem., ***279(32)***, *33220-33227 (2004)*

Wnt signaling pathways regulate many developmental responses; however, little is known about how Wnt ligands function on a biochemical level. Recent studies have shown that Wnt-3a is palmitoylated before secretion. Here we report that *Drosophila* Wnt-1 (Wingless) also undergoes a lipid modification. Lipidation occurs in the endoplasmic reticulum and is dependent on Porcupine, a putative *O*-acyltransferase. After modification, DWnt-1 partitions as a membrane-anchored protein and is sorted into lipid raft detergent-insoluble microdomains. Lipidation, raft targeting, and secretion can be blocked by the addition of 2-bromopalmitate, a competitive inhibitor of *O*-acyltransferase activity. Based on these results we propose a model whereby lipidation targets Wnt-1 to secretory vesicles that deliver the ligand to specialized microdomains at the cell surface where it can be packaged for secretion.

3.522 Oligomerization of the sensory and motor neuron-derived factor prevents protein O-glycosylation
Cabedo, H., Carteron, C. and Ferrer-Montiel, A.
J. Biol. Chem., ***279(32)***, *33623-33629 (2004)*

The sensory and motor neuron-derived factor (SMDF) is a neuregulin that promotes Schwann cell proliferation and differentiation. Hence, understanding axon myelination is important to unveil the mechanisms involved in SMDF biogenesis, membrane delivery, and compartmentalization. SMDF is a type II membrane protein expressed as two distinct polypeptides of ~40 and 83 kDa. Whether the 83-kDa polypeptide results from posttranslational modifications of the protein monomers or protein dimerization remains unknown. Here we have addressed this question and shown that the 83-kDa polypeptide is an *O*-glycosylated form of the protein. Deletion of the N-terminal domain fully abrogates the SMDF *O*-glycosylation, indicating that incorporation of *O*-glycans occurs in the intracellular domain of the protein. Notably, *O*-glycosylated forms are excluded from partitioning into lipid raft microdomains. In addition, we found that heterologously expressed SMDF monomers interact in intact living cells as evidenced from fluorescence resonance energy transfer of cyan fluorescent protein/yellow fluorescent protein·SMDF

fusion proteins. A stepwise deletion approach demonstrated that SMDF self-association is primarily determined by its transmembrane segment. Notably, biochemical analysis revealed that SMDF multimers are exclusively composed of the 40-kDa polypeptide. Collectively, these findings indicate that the 40-kDa form corresponds to unmodified SMDF, which may be present as multimers, whereas the 83-kDa polypeptide is a monomeric *O*-glycosylated form of the protein. Furthermore, our observations imply a role for oligomerization as a potential modulator of the distribution in membrane domains and *O*-glycosylation of the protein.

3.523 Differential signaling pathways are activated in the Epstein-Barr virus-activated malignancies nasopharyngeal carcinoma and Hodgkin lymphoma
Morrison, J.A., Gulley, M.L., Pathmanathan, R. and Raab-Traub, N.
*Cancer. Res.,**64**, 5251-5260 (2004)*

EBV is associated with the epithelial cancer, nasopharyngeal carcinoma (NPC), and the lymphoid malignancy, Hodgkin lymphoma (HL). The EBV latent membrane proteins 1 and 2A are expressed in these tumors. These proteins activate the phosphatidylinositol 3'-OH kinase (PI3K)/Akt pathway, which is commonly activated inappropriately in malignancy. In this study, the status of Akt activation and its targets, glycogen synthase kinase-3ß (GSK-3ß) and ß-catenin, was investigated in NPC and HL clinical specimens. In the majority of HL and NPC specimens, Akt was activated, indicating an important role for this kinase in the development and/or progression of these tumors. Akt phosphorylates and inactivates GSK-3ß, a negative regulator of the proto-oncoprotein ß-catenin that is aberrantly activated in many cancers. GSK-3ß was phosphorylated and inactivated with concomitant nuclear ß-catenin accumulation in the majority of NPC specimens. The malignant cells of the majority of HL cases, however, did not have inactivated GSK-3ß and lacked nuclear ß-catenin expression. These data indicate that this signaling arm of PI3K/Akt is universal and important in NPC pathogenesis but is apparently not affected in HL. These findings point to a divergence in pathways activated by EBV in different cellular contexts.

3.524 Comparisons of physical separation methods of Kunjin virus-induced membranes
Kim, M., Mackenzie, J.M. and Westaway, E.G.
*J. Virol. Meth., **120**, 179-187 (2004)*

two sets of connected membranes induced in Kunjin virus-infected cells are characterized by the presence of NS3 helicase/protease in both, and by RNA-dependent RNA polymerase (RdRp) activity plus the associated double-stranded RNA (dsRNA) template in vesicle packets (VP), or by the absence of both the VP-specific markers in the convoluted membranes/paracrystalline arrays (CM/PC). Attempts were made to separate flavivirus-induced membranes by sedimentation or flotation analyses in density gradients of sucrose or **iodixanol,** respectively, after treatment of cell lysates by sonication, osmotic shock, or tryptic digestion. Only osmotic shock treatment provided suggestive evidence of separation. This was explored by flow cytometry analysis (FCA) of RdRp active membrane fractions from a sucrose gradient, using dual fluorescent labelling via antibodies to NS3 and dsRNA. FCA revealed the presence of a dual labelled membrane population indicative of VP, and in a faster sedimenting fraction a membrane population able to be labelled only in NS3, representative of CM/PC and associated (R)ER. It was postulated that osmotic shock ruptured the bounding membrane of the VP, releasing the enclosed small vesicles associated with the Kunjin virus replication complex characterized previously. Notably, the presence of the full spectrum of nonstructural proteins in some membrane fractions was not a reliable marker for RdRp activity. These experiments may provide the opportunity for isolation of relatively pure flavivirus replication complexes in their native membrane-associated state by fluorescence-activated cell sorting.

3.525 Membrane dynamics in giant unilamminar vesicles (GUVS) of raft and non-raft fractions of brushborders membranes
Cheng, M. et al
Biophysical Meeting 2004 abstract

Lipid rafts are discrete regions in the plasma membrane which are composed of lipids that exist in the liquid ordered state, and are believed to function as platforms for protein and lipid transport. These structures are reported to be present in numerous membrane systems, including brushborder membranes in kidney cells. This research aims to study the membrane dynamics in brushborder membranes and membrane fractions of rat renal proximal tubular cells. Fluorescence (Laurdan) provided direct visualization of GUVs formed through electroformation of raft and non-raft fractions of intact renal brushborder membranes. Flotation through a density gradient (Optiprep) separated the raft and non-raft

fractions without the use of a detergent. Two-photon scanning microscopy of the GUVs formed from the raft fraction showed uniform fluorescence intensity images with some non-fluorescent domains of a few microns in size. Previous GUV studies of raft fractions obtained through detergent extraction yielded vesicles devoid of domains. Membrane fluidity measured as Laurdan Generalized Polarization (GP) function was monitored across a physiological temperature range (25°-42°C) in the GUVs. The GP in the raft fraction indicated a less fluid phase than in the non-raft fraction. This result is consistent with the compositional differences in these membrane fractions, particularly in terms of sphingomyelin and cholesterol content. Scanning fluctuation correlation spectroscopy performed on the intact brushborder membrane revealed the diffusion rate of NaPi-II cotransporter, a brushborder membrane specific protein, to be consistent with the mobility of a membrane associated protein.

3.526 Phagosome microdomains define foci of specialized functions in innate immunity
Goyette, G. et al
ICI/FOCIS 2004 meeting abstract no. 2246 (2004)

Macrophages are cells of the immune system specialized in the destruction of invading pathogens, and the elaboration of an efficient immune response. Phagocytosis, the process by which pathogens are internalized by host cells, leads to the formation of phagosomes at the cell surface by the direct recruitment of the endoplasmic reticulum. Phagosomes engage in a complex maturation process leading to the formation of phagolysosomes, allowing the killing and degradation of microorganisms. Unfortunately, several microorganisms have evolved strategies to alter phagolysosome biogenesis, a process still poorly understood. Proteomics analyses revealed that phagosomes are made of hundreds of proteins, highlighting the complexity of the molecular mechanisms involved in phagosome functions. We have shown recently that the phagosome membrane is not homogeneous but rather made of microdomains. The functions of these cholesterol-enriched microdomains on phagosomes are unknown. To understand these functions, we initiated the systematic characterization of these domains using a proteomics approach. Phagosome microdomains were isolated based on their insolubility in Triton X-100 and their flotation on Optiprep™ step gradients. MS/MS analyses of these microdomains led to the identification of 264 proteins indicating that functions such as acidification, cholesterol metabolism, signal transduction, and membrane fusion are likely to take place on phagosome membrane microdomains.
Results also indicate that the intracellular pathogen *Leishmania donovani* survives in macrophages by using a surface glycolipid to disrupt the proper organization of phagosome lipid rafts, highlighting the potential involvement of these structures in our ability to fight infection.

3.527 Linking receptor-mediated endocytosis and cell signaling
Zou, Z. et al
J. Biol. Chem., ***279(33)***, *34302-34310 (2004)*

Megalin, a member of the low density lipoprotein receptor gene family, is required for efficient protein absorption in the proximal tubule. Recent studies have shown that the low density lipoprotein receptor-related protein, another member of this gene family, is proteolytically processed by γ-secretase implying a role for low density lipoprotein receptor-related protein in a Notchlike signaling pathway. This pathway has been shown to involve: 1) metalloprotease-mediated ectodomain shedding and γ-secretase-mediated intramembrane proteolysis of some receptors. Experiments were performed to determine whether megalin undergoes similar processing. By immunocytochemistry, immunoblotting, and a fluorogenic enzyme assay presenilin-1 (required for γ-secretase activity) and γ-secretase activity were found in the brush border of proximal kidney tubules where megalin is localized. Using a fluorogenic peptide containing an amyloid precursor protein γ-secretase cleavage site and Compound E, a specific γ-secretase inhibitor, we found high levels of γ-secretase activity in renal brush border membrane vesicles. Immunoblotting analysis of renal microsomes and opossum kidney proximal tubule (OKP) cells using antibodies directed to the cytosolic domain of megalin showed a 35–40-kDa, membrane-associated, carboxyl-terminal fragment of megalin (MCTF). When cells were incubated with 200 nM phorbol 12-myristate 13-acetate, the appearance of the MCTF increased 2.5-fold and was blocked by metalloprotease inhibitors. When the cells were incubated with γ-secretase inhibitor Compound E, it caused a 2-fold increase in MCTF. Finally, incubating the cells with 1 μM vitamin D-binding protein resulted in a 25% increase in the appearance of the MCTF. In summary, the MCTF is produced by protein kinase C regulated, metalloprotease-mediated ectodomain shedding and is the substrate for γ-secretase. We postulate that the enzymatic processing of megalin represents part of a novel ligand-dependent signaling pathway in the proximal tubule that links receptor-mediated endocytosis with cell signaling.

3.528 Bph1p, the *Saccharomyces cerevisiae* homologue of CHS1/beige, functions in cell wall formation and protein sorting

Shiffert, S.L., Vaughn, M.B., Huynh, D., Kaplan, J. and McVey Ward, D.
*Traffic, **5**, 700-710 (2004)*

Mutations in the Chediak-Higashi syndrome gene (*CHS1*) and its murine homologue *Beige* result in the formation of enlarged lysosomes. *BPH1* (Beige Protein Homologue 1) encodes the *Saccharomyces cerevisiae* homologue of CHS1/Beige. *BPH1* is not essential and the encoded protein was found to be both cytosolic and peripherally bound to a membrane. Neither disruption nor overexpression of *BPH1* affected vacuole morphology as assessed by fluorescence microscopy. The δ*bph1* strain showed an impaired growth on defined synthetic media containing potassium acetate buffered below pH 4.25, increased sensitivity to calcofluor white, and increased agglutination in response to low pH. A library screen identified *VPS9*, *FLO1*, *FLO9*, *BTS1* and *OKP1* as high copy suppressors of the growth defect of δ*bph1* on both low pH potassium acetate and calcofluor white. The δ*bph1* strain demonstrated a mild defect in sorting vacuolar components, including increased secretion of carboxypeptidase Y and missorting of alkaline phosphatase. Overexpression of *VPS9*, *BTS1* and *OKP1* suppressed the carboxypeptidase Y secretion defect of δ*bph1*. Overexpression of *BPH1* was found to suppress the calcofluor white sensitivity of a class E *VPS* deletion strain, δ*vta1*. Together, these data suggest that Bph1p associates with a membrane and is involved in protein sorting and cell wall formation.

3.529 Nicalin and its binding partner Nomo are novel Nodal aignaling antagonists

Haffner, C. et al

Nodals are signaling factors of the transforming growth factor-ß (TGFß) superfamily with a key role in vertebrate development. They control a variety of cell fate decisions required for the establishment of the embryonic body plan. We have identified two highly conserved transmembrane proteins, Nicalin and Nomo (Nodal modulator, previously known as pM5), as novel antagonists of Nodal signaling. Nicalin is distantly related to Nicastrin, a component of the Alzheimer's disease-associated γ-secretase, and forms a complex with Nomo. Ectopic expression of both proteins in zebrafish embryos causes cyclopia, a phenotype that can arise from a defect in mesendoderm patterning mediated by the Nodal signaling pathway. Accordingly, downregulation of Nomo resulted in an increase in anterior axial mesendoderm and the development of an enlarged hatching gland. Inhibition of Nodal signaling by ectopic expression of Lefty was rescued by reducing Nomo levels. Furthermore, Nodal- as well as Activin-induced signaling was inhibited by Nicalin and Nomo in a cell-based reporter assay. Our data demonstrate that the Nicalin/Nomo complex antagonizes Nodal signaling during mesendodermal patterning in zebrafish.

3.530 Cell surface ceramide generation precedes and controls FcγRII clustering and phosphorylation in rafts

Shakor, A.B.A., Kwiatkowska, K. and Sobota, A.
*J. Biol. Chem., **279(35)**, 36778-36787 (2004)*

Despite the role of sphingolipid/cholesterol rafts as signaling platforms for Fcγ receptor II (FcγRII), the mechanism governing translocation of an activated receptor toward the rafts is unknown. We show that at the onset of FcγRII cross-linking acid sphingomyelinase is rapidly activated. This enzyme is extruded from intracellular compartments to the cell surface, and concomitantly, exofacially oriented ceramide is produced. Both non-raft and, to a lesser extent, raft sphingomyelin pools were hydrolyzed at the onset of FcγRII cross-linking. The time course of ceramide production preceded the recruitment of FcγRII to rafts and the receptor phosphorylation. Exogenous C_{16}-ceramide facilitated clustering of FcγRII and its association with rafts. In contrast, inhibition of acid sphingomyelinase diminished both the ceramide generation and clustering of cross-linked FcγRII. Under these conditions, tyrosine phosphorylation of FcγRII and receptor-accompanying proteins was also reduced. All the inhibitory effects were bypassed by treatment of cells with exogenous ceramide. These data provide evidence that the generation of cell surface ceramide is a prerequisite for fusion of cross-linked FcγRII and rafts, which triggers the receptor tyrosine phosphorylation and signaling.

3.531 Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells

Seyrantepe, V. et al
*J. Biol. Chem., **279(35)**, 37021-37029 (2004)*

Three different mammalian sialidases have been described as follows: lysosomal (Neu1, gene *NEU1*), cytoplasmic (Neu2, gene *NEU2*), and plasma membrane (Neu3, gene *NEU3*). Because of mutations in the *NEU1* gene, the inherited deficiency of Neu1 in humans causes the severe multisystemic neurodegenerative disorder sialidosis. Galactosialidosis, a clinically similar disorder, is caused by the secondary Neu1 deficiency because of genetic defects in cathepsin A that form a complex with Neu1 and activate it. In this study we describe a novel lysosomal lumen sialidase encoded by the *NEU4* gene on human chromosome 2. We demonstrate that Neu4 is ubiquitously expressed in human tissues and has broad substrate specificity by being active against sialylated oligosaccharides, glycoproteins, and gangliosides. In contrast to Neu1, Neu4 is targeted to lysosomes by the mannose 6-phospate receptor and does not require association with other proteins for enzymatic activity. Expression of Neu4 in the cells of sialidosis and galactosialidosis patients results in clearance of storage materials from lysosomes suggesting that Neu4 may be useful for developing new therapies for these conditions.

3.532 Atg21 is required for effective recruitment of Atg8 to the preautophagosomal structure during the Cvt pathway
Meiling-Wesse, K. et al
J. Biol. Chem., ***279(36)***, *37741-37759 (2004)*

Atg21 and Atg18 are homologue yeast proteins. Whereas Atg18 is essential for the Cvt pathway and autophagy, a lack of Atg21 only blocks the Cvt pathway. Our proteinase protection experiments now demonstrate that growing *atg21*Δ cells fail to form proaminopeptidase I-containing Cvt vesicles. Quantitative measurement of autophagy in starving *atg21*Δ cells showed only 35% of the wild-type rate. This suggests that Atg21 plays a nonessential role in improving the fidelity of autophagy. The intracellular localization of Atg21 is unique among the Atg proteins. In cells containing multiple vacuoles, Atg21-yellow fluorescent protein clearly localizes to the vertices of the vacuole junctions. Cells with a single vacuole show most of the protein at few perivacuolar punctae. This distribution pattern is reminiscent to the Vps class C(HOPS) (homotypic fusion and vacuolar protein sorting) protein complex. In growing cells, Atg21 is required for effective recruitment of Atg8 to the preautophagosomal structure. Consistently, the covalent linkage of Atg8 to the lipid phosphatidylethanolamine is significantly retarded. Lipidated Atg8 is supposed to act during the elongation of autophagosome precursors. However, despite the reduced autophagic rate and the retardation of Atg8 lipidation, electron microscopy of starved *atg21*Δ *ypt7*Δ double mutant cells demonstrates the formation of normally sized autophagosomes with an average diameter of 450 nm.

3.533 Interactions of EGFR and caveolin-1 in human glioblastoma cells: evidence that tyrosine phosphorylation regulates EGFR association with caveolae
Abulrob, A. et al
Oncogene, ***23****, 6967-6979 (2004)*

Epidermal growth factor receptor (EGFR) amplification and type III mutation (EGFRvIII), associated with constitutive tyrosine kinase activation and high malignancy, are commonly observed in glioblastoma tumors. The association of EGFR and EGFRvIII with caveolins was investigated in human glioblastoma cell lines, U87MG and U87MG-EGFRvIII. Caveolin-1 expression, determined by RT–PCR, real-time quantitative PCR and Western blot, was upregulated in glioblastoma cell lines (two-fold) and tumors (20–300-fold) compared to primary human astrocytes and nonmalignant brain tissue, respectively. U87MG-EGFRvIII expressed higher levels of caveolin-1 than U87MG. In contrast, the expression of caveolin-2 and -3 were downregulated in glioblastoma cells compared to astrocytes. A colocalization of EGFR, but not of EGFRvIII, with lipid rafts and caveolin-1 was observed by immunocytochemistry. Association of EGFR and EGFRvIII with caveolae, assessed *in vitro* by binding to caveolin scaffolding domain peptides and *in vivo* by immunocolocalization studies in cells and caveolae-enriched cellular fraction, was phosphorylation-dependent: ligand-induced phosphorylation of EGFR resulted in dissociation of EGFR from caveolae. In contrast, inhibition of the EGFRvIII constitutive tyrosine phosphorylation by AG1478 increased association of EGFRvIII with caveolin-1. AG1478 also increased caveolin-1 expression and reduced glioblastoma cell growth in a semi-solid agar. The evidence suggests that the phosphorylation-regulated sequestration of EGFR in caveolae may be involved in arresting constitutive or ligand-induced signaling through EGFR responsible for glial cell transformation.

3.534 Isolated plant nuclei as mechanical and thermal sensors involved in calcium signaling
Xiong, T.C., Jauneau, A., Ranjeva, R. and Mazars, C.
The Plant J., ***40****, 12-21 (2004)*

Calcium signals in the nucleus elicit downstream effects that are distinct from those of cytosolic calcium signals. In the present work, we have evaluated the ability of plant nuclei to sense stimuli directly and to convert them into calcium changes. We show that individual mechanical stimulation of isolated nuclei elicits a single calcium transient at acidic pHs, whereas a series of stimulations leads to oscillations whose frequency reflects that of the stimuli. Conversely, at alkaline pHs, nuclei respond to temperature but not to stretch. The stretch- and the temperature-activated processes differ by their sensitivity to pharmacological drugs known to affect ion channel activities in animal cells. Our data demonstrate that isolated nuclei are able to gauge physical parameters of their environment. This might have a profound influence on the functioning of calcium-dependent processes known to control a large array of molecular events in the nucleus.

3.535 CLIPR-59 is a lipid raft-associated protein containing a cytoskeleton-associated protein glycine-rich domain (CAP-Gly) that perturbs microtubule dynamics
Lallamand-Breitenbach, V. et al
J. Biol. Chem., ***279(39)***, *41168-41178 (2004)*

We recently have identified a new cytoplasmic linker protein (CLIP), CLIPR-59, which is involved in the regulation of early endosome/*trans*-Golgi network dynamics. In contrast with CLIP-170, CLIPR-59 is not localized to microtubules at steady state but is associated with the *trans*-Golgi network and the plasma membrane. Here we show that the last 30 amino acids (C30) are sufficient for membrane targeting and that two cysteines in the C30 domain are palmitoylated. We demonstrate that CLIPR-59 is associated with lipid rafts via its C-terminal palmitoylated domain. *In vitro* experiments suggest that CLIPR-59 and its microtubule-binding domain alone have a better affinity for unpolymerized tubulin or small oligomers than for microtubules. In contrast with the CLIP-170 microtubule-binding domain, the CLIPR-59 microtubule-binding domain diminishes microtubule regrowth after nocodazole washout *in vivo*, showing that this domain can prevent microtubule polymerization. In contrast with the role of linker between membranes and microtubules that was proposed for CLIP function, CLIPR-59 thus may have an "anti-CLIP" function by preventing microtubule-raft interactions.

3.536 Reorganization of lipid rafts during capacitation of human sperm
Cross, N.L.
Biol. Reprod., ***71***, *1367-1373 (2004)*

Ejaculated mammalian sperm must complete a final maturation, termed capacitation, before they can undergo acrosomal exocytosis and fertilize an egg. In human sperm, loss of sperm sterol is an obligatory, early event in capacitation. How sterol loss leads to acrosomal responsiveness is unknown. These experiments tested the hypothesis that loss of sperm sterol affects the organization of cold detergent-resistant membrane microdomains (lipid "rafts"). The GPI-linked protein CD59, the ganglioside GM1, and the protein flotillin-2 were used as markers for lipid rafts. In uncapacitated sperm, 51% of the CD59, 41% of the GM1, and 90% of the flotillin-2 were found in the raft fraction. During capacitation, sperm lost 67% of their 3ß-hydroxysterols, and the percentages of CD59 and GM1 in the raft fraction decreased to 34% and 31%, respectively. The distribution of flotillin-2 did not change. Preventing a net loss of sperm sterol prevented the loss of CD59 and GM1 from the raft fraction. Fluorescence microscopy showed CD59 and GM1 to be distributed over the entire sperm surface. Flotillin-2 was located mainly in the posterior head and midpiece. Patching using bivalent antibodies indicated that little of the GM1 and CD59 was stably associated in the same membrane rafts. Likewise, GM1 and flotillin-2 were not associated in the same membrane rafts. In summary, lipid rafts of heterogeneous composition were identified in human sperm and the two raft components, GM1 and CD59, showed a partial sterol loss-dependent shift to the nonraft domain during capacitation.

3.537 An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and RNA replication
Elazar, M., Liu, P., Rice, C.M. and Glenn, J.S.
J. Virol., ***78(20)***, *11393-11400 (2004)*

Like other positive-strand RNA viruses, hepatitis C virus (HCV) is believed to replicate its RNA in association with host cell cytoplasmic membranes. Because of its association with such membranes, NS4B, one of the virus's nonstructural proteins, may play an important role in this process, although the mechanistic details are not well understood. We identified a putative N-terminal amphipathic helix (AH) in

NS4B that mediates membrane association. Introduction of site-directed mutations designed to disrupt the hydrophobic face of the AH abolishes the AH's ability to mediate membrane association. An AH in NS4B is conserved across HCV isolates. Completely disrupting the amphipathic nature of NS4B's N-terminal helix abolished HCV RNA replication, whereas partial disruption resulted in an intermediate level of replication. Finally, immunofluorescence studies revealed that HCV replication complex components were mislocalized in the AH-disrupted mutant. These results identify a key membrane-targeting domain which can form the basis for developing novel antiviral strategies.

3.538 Functional similarity between the peroxisomal PTS2 receptor binding protein Pex18p and the N-terminal half of the PTS1 receptor Pex5p
Schäfer, A., Kerssen, D., Veenhuis, M., Kunau, W-H. And Schliebs, W.
*Mol. Cell Biol., **24(20)**, 8895-8906 (2004)*

Within the extended receptor cycle of peroxisomal matrix import, the function of the import receptor Pex5p comprises cargo recognition and transport. While the C-terminal half (Pex5p-C) is responsible for PTS1 binding, the contribution of the N-terminal half of Pex5p (Pex5p-N) to the receptor cycle has been less clear. Here we demonstrate, using different techniques, that in *Saccharomyces cerevisiae* Pex5p-N alone facilitates the import of the major matrix protein Fox1p. This finding suggests that Pex5p-N is sufficient for receptor docking and cargo transport into peroxisomes. Moreover, we found that Pex5p-N can be functionally replaced by Pex18p, one of two auxiliary proteins of the PTS2 import pathway. A chimeric protein consisting of Pex18p (without its Pex7p binding site) fused to Pex5p-C is able to partially restore PTS1 protein import in a *PEX5* deletion strain. On the basis of these results, we propose that the auxiliary proteins of the PTS2 import pathway fulfill roles similar to those of the N-terminal half of Pex5p in the PTS1 import pathway.

3.539 BZLF1, an Epstein-Barr virus immediate-early protein, induces p65 nuclear translocation while inhibiting p65 transcriptional function
Morrison, T.E. and Kenncy, S.C.
*Virology, **328**, 219-232 (2004)*

We have previously demonstrated that the Epstein–Barr virus immediate–early BZLF1 protein interacts with, and is inhibited by, the NF-κB family member p65. However, the effects of BZLF1 on NF-κB activity have not been intensively studied. Here we show that BZLF1 inhibits p65-dependent gene expression. BZLF1 inhibited the ability of IL-1, as well as transfected p65, to activate the expression of two different NF-κB-responsive genes, ICAM-1 and IκB-α. BZLF1 also reduced the constitutive level of IκB-α protein in HeLa and A549 cells, and increased the amount of nuclear NF-κB to a similar extent as tumor necrosis factor-alpha (TNF-α) treatment. In spite of this BZLF1-associated increase in the nuclear form of NF-κB, BZLF1 did not induce binding of NF-κB to NF-κB responsive promoters (as determined by chromatin immunoprecipitation assay) in vivo, although TNF-α treatment induced NF-κB binding as expected. Overexpression of p65 dramatically inhibited the lytic replication cycle of EBV in 293-EBV cells, confirming that NF-κB also inhibits BZLF1 transcriptional function. Our results are consistent with a model in which BZLF1 inhibits the transcriptional function of p65, resulting in decreased transcription of IκB-α, decreased expression of IκB-α protein, and subsequent translocation of NF-κB to the nucleus. This nuclear translocation of NF-κB may promote viral latency by negatively regulating BZLF1 transcriptional activity. In situations where p65 activity is limiting in comparison to BZLF1, the ability of BZLF1 to inhibit p65 transcriptional function may protect the virus from the host immune system during the lytic form of infection.

3.540 Targeting, import, and dimerization of a mammalian mitochondrial ATP binding cassette (ABC) transporter, ABCB10 (ABC-me)
Graf, S.A., Haigh, S.E., Corson, E.D. and Shirihai, O.S.
*J. Biol. Chem., **279(41)**, 42954-42963 (2004)*

ATP binding cassette (ABC) transporters are a diverse superfamily of energy-dependent membrane translocases. Although responsible for the majority of transmembrane transport in bacteria, they are relatively uncommon in eukaryotic mitochondria. Organellar trafficking and import, in addition to quaternary structure assembly, of mitochondrial ABC transporters is poorly understood and may offer explanations for the paucity of their diversity. Here we examine these processes in ABCB10 (ABC-me), a

mitochondrial inner membrane erythroid transporter involved in heme biosynthesis. We report that ABCB10 possesses an unusually long 105-amino acid mitochondrial targeting presequence (mTP). The central subdomain of the mTP (amino acids (aa) 36–70) is sufficient for mitochondrial import of enhanced green fluorescent protein. The N-terminal subdomain (aa 1–35) of the mTP, although not necessary for the trafficking of ABCB10 to mitochondria, participates in the proper import of the molecule into the inner membrane. We performed a series of amino acid mutations aimed at changing specific properties of the mTP. The mTP requires neither arginine residues nor predictable α-helices for efficient mitochondrial targeting. Disruption of its hydrophobic character by the mutation L46Q/I47Q, however, greatly diminishes its efficacy. This mutation can be rescued by cryptic downstream (aa 106–715) mitochondrial targeting signals, highlighting the redundancy of this protein's targeting qualities. Mass spectrometry analysis of chemically cross-linked, immunoprecipitated ABCB10 indicates that ABCB10 embedded in the mitochondrial inner membrane homodimerizes and homo-oligomerizes. A deletion mutant of ABCB10 that lacks its mTP efficiently targets to the endoplasmic reticulum. Quaternary structure assembly of ABCB10 in the ER appears to be similar to that in the mitochondria.

3.541 Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus
Hood, J.L., Brooks, W.H. and Roszman, T.L.
J. Biol. Chem., ***279(41)****, 43126-43135 (2004)*

Calpain, a calcium-activated cysteine protease, is involved in modulating a variety of cell activities such as shape change, mobility, and apoptosis. The two ubiquitous isoforms of this protease, calpain I and II, are considered to be cytosolic proteins that can translocate to various sites in the cell. The activity of calpain is modulated by two regulatory proteins, calpastatin, the specific endogenous inhibitor of calpain, and the 28-kDa regulatory subunit. Using velocity gradient centrifugation, the results of this study confirm and greatly expand upon our previous finding that the calpain/calpastatin network is associated with the endoplasmic reticulum and Golgi apparatus in cells. Moreover, confocal microscopy demonstrates that calpain II colocalizes with specific proteins found in these organelles. Additional experiments reveal that hydrophobic rather than electrostatic interactions are responsible for the association of the calpain/calpastatin network with these organelles. Treatment of the organelles with Na_2CO_3 or deoxycholate reveal that calpain I, 78-kDa calpain II, and the regulatory subunit are "embedded" within the organelle membranes similar to integral membrane proteins. Proteinase K treatment of the organelles shows that calpain I and II, calpastatin, and the regulatory subunit localize to the cytosolic surface of the organelle membranes, and a subset of calpain II and the regulatory subunit are also found within the lumen of these organelles. These results provide a new and novel explanation for how the calpain/calpastatin network is organized in the cell.

3.542 Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts
Matto, M., Rice, C.M., Aroeti, B. and Glenn, J.S.
J. Virol., ***78(21)****, 12047-12053 (2004)*

A subpopulation of hepatitis C virus (HCV) core protein in cells harboring full-length HCV replicons is biochemically associated with detergent-resistant membranes (DRMs) in a manner similar to that of markers of classical lipid rafts. Core protein does not, however, colocalize in immunofluorescence studies with classical plasma membrane raft markers, such as caveolin-1 and the B subunit of cholera toxin, suggesting that core protein is bound to cytoplasmic raft microdomains distinct from caveolin-based rafts. Furthermore, while both the structural core protein and the nonstructural protein NS5A associate with membranes, they do not colocalize in the DRMs. Finally, the ability of core protein to localize to the DRMs did not require other elements of the HCV polyprotein. These results may have broad implications for the HCV life cycle and suggest that the HCV core may be a valuable probe for host cell biology.

3.543 Dynamic confinement of NK2 receptors in the plasma membrane
Cezanne, L. et al
J. Biol. Chem., ***279(43)****, 45057-45067 (2004)*

A functional fluorescent neurokinin NK2 receptor, EGFP-NK2, was previously used to follow, by fluorescence resonance energy transfer measurements in living cells, the binding of its fluorescently labeled agonist, bodipy-neurokinin A (NKA). Local agonist application suggested that the activation and desensitization of the NK2 receptors were compartmentalized at the level of the plasma membrane. In this

study, fluorescence recovery after photobleaching experiments are carried out at variable observation radius (vrFRAP) to probe EGFP-NK2 receptor mobility and confinement. Experiments are carried out at 20 °C to maintain the number of receptors constant at the cell surface during recordings. In the absence of agonist, 35% EGFP-NK2 receptors diffuse within domains of 420 ± 80 nm in radius with the remaining 65% of receptors able to diffuse with a long range lateral diffusion coefficient between the domains. When cells are incubated with a saturating concentration of NKA, 30% EGFP-NK2 receptors become immobilized in small domains characterized by a radius equal to 170 ± 50 nm. Biochemical experiments show that the confinement of EGFP-NK2 receptor is not due to its association with rafts at any given time. Colocalization of the receptor with β-arrestin and transferrin supports that the small domains, containing 30% of activated EGFP-NK2, correspond to clathrin-coated pre-pits. The similar amount of confined EGFP-NK2 receptors found before and after activation (30–35%) is discussed in term of putative transient interactions of the receptors with preexisting scaffolds of signaling molecules.

3.544 ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulatd sterol movement from the plasma membrane to the endoplasmic reticulum
Li, Y and Priz, W.A.
J. Biol. Chem., ***279(43)***, *45226-45234 (2004)*

Little is known about the mechanisms of intracellular sterol transport or how cells maintain the high sterol concentration of the plasma membrane (PM). Here we demonstrate that two inducible ATP-binding cassette (ABC) transporters (Aus1p and Pdr11p) mediate nonvesicular movement of PM sterol to the endoplasmic reticulum (ER) in *Saccharomyces cerevisiae*. This transport facilitates exogenous sterol uptake, which we find requires steryl ester synthesis in the ER. Surprisingly, while expression of Aus1p and Pdr11p significantly increases sterol movement from PM to ER, it does not alter intracellular sterol distribution. Thus, ER sterol is likely rapidly returned to the PM when it is not esterified in the ER. We show that the propensity of PM sterols to be moved to the ER is largely determined by their affinity for sterol sphingolipid-enriched microdomains (rafts). Our findings suggest that raft association is a primary determinant of sterol accumulation in the PM and that Aus1p and Pdr11p facilitate sterol uptake by increasing the cycling of sterol between the PM and ER.

3.545 Participation of G protein in natriuretic peptide hormone secretion from heart atria
Bensimon, M. et al
Endocrinol., ***145***, *5313-5321 (2004)*

The involvement of G proteins in the mechanism underlying the increased atrial natriuretic factor (ANF) secretion observed after atrial muscle stretch (stretch-secretion coupling) was assessed using a combined pharmacological, immunocytochemical, and tissue fractionation approach. It was found that $G_{i/o}$ inhibition by pertussis toxin (PTX) abolished stretch-secretion coupling without affecting baseline secretion through a mechanism that is independent of G_q signaling agonists. Mastoparan-7, a $G_{i/o}$ agonist, significantly increased ANF secretion even in the absence of muscle stretch through a PTX-sensitive mechanism. By confocal and electron immunocytochemistry, ANF and G_o partially colocalized, whereas ultracentrifugation analysis suggested the presence of two populations of granules, one of which was partially associated with G_o, as demonstrated by Western blotting. PTX did not affect basal or endothelin-1-stimulated ANF secretion, in line with the view that endothelin-1 signals mainly through G_q. It is concluded there are at least two types of regulated secretory processes in atrial cardiocytes: one is acutely responsive to muscle stretch and is PTX sensitive, and the other is $G_q$mediated and PTX insensitive and may be responsible for changes in secretion after chronic changes in the neuroendocrine environment.

3.546 Real time analysis of intact organelle using surface plasmon resonance
Ferraci, G., Seagar, M., Joël, C., Miquelis, R. And Leveque, C.
Anal. Biochem., ***334***, *367-375(2004)*

Membrane proteins remain refractory to standard protein chip analysis. They are typically expressed at low densities in distinct subcellular compartments, their biological activity can depend on assembly into macromolecular complexes in a specific lipid environment. We report here a real-time, label-free method to analyze membrane proteins inserted in isolated native synaptic vesicles. Using surface plasmon resonance-based biomolecular interaction analysis (Biacore), organelle capture from minute quantities of 10,000*g* brain supernatant (1–10 μg) was monitored. Immunological and morphological characterization indicated that pure intact synaptic vesicles were immobilized on sensor chips. Vesicle chips were stable for days, allowing repetitive use with multiple analytes. This method provides an efficient way in which to

characterize organelle membrane components in their native context. Organelle chips allow a broad range of measurements, including interactions of exogenous ligands with the organelle surface (kinetics, K_d), and protein profiling.

3.547 The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1α,25(OH)$_2$-vitamin D$_3$ in vivo and in vitro

Huhtakangas, J.A., Olivera, C.J., Bishop, J.E., Zanello, L.P. and Norman, A.W.
Mol. Endocrinol., ***18(11)***, *2660-2671 (2004)*

The steroid hormone 1α,25(OH)$_2$-vitamin D$_3$ (1,25D) regulates gene transcription through a nuclear receptor [vitamin D receptor (VDR)] and initiation of rapid cellular responses through a putative plasma membrane-associated receptor (VDR$_{mem}$). This study characterized the VDR$_{mem}$ present in a caveolae-enriched membrane fraction (CMF), a site of accumulation of signal transduction agents. Saturable and specific [^{3}H]-1,25D binding *in vitro* was found in CMF of chick, rat, and mouse intestine; mouse lung and kidney; and human NB4 leukemia and rat ROS 17/2.8 osteoblast-like cells; in all cases the 1,25D K_D binding dissociation constant = 1–3 nM. Our data collectively support the classical VDR being the VDR$_{mem}$ in caveolae: 1) VDR antibody immunoreactivity was detected in CMF of all tissues tested; 2) competitive binding of [^{3}H]-1,25D by eight analogs of 1,25D was significantly correlated between nuclei and CMF (r^2 = 0.95) but not between vitamin D binding protein (has a different ligand binding specificity) and CMF; 3) confocal immunofluorescence microscopy of ROS 17/2.8 cells showed VDR in close association with the caveolae marker protein, caveolin-1, in the plasma membrane region; 4) *in vivo* 1,25D pretreatment reduced *in vitro* [^{3}H]-1,25D binding by 30% in chick and rat intestinal CMF demonstrating *in vivo* occupancy of the CMF receptor by 1,25D; and 5) comparison of [^{3}H]-1,25D binding in VDR KO and WT mouse kidney tissue showed 85% reduction in VDR KO CMF and 95% reduction in VDR KO nuclear fraction. This study supports the presence of VDR as the 1,25D-binding protein associated with plasma membrane caveolae.

3.548 Both the sequence and length of the C terminus of PEN-2 are critical for intermolecular interactions and function of presenilin complexes

Hasegawa, H. et al
J. Biol. Chem., ***279(45)***, *46455-46463 (2004)*

Presenilin 1 or presenilin 2, nicastrin, APH-1, and PEN-2 form high molecular weight complexes that play a pivotal role in the cleavage of various Type I transmembrane proteins, including the β-amyloid precursor protein. The specific function of PEN-2 is unclear. To explore its function and intermolecular interactions, we conducted deletion and mutagenesis studies on a series of conserved residues at the C terminus of PEN-2. These studies suggest that: 1) both the presence and amino acid sequence of the conserved DYLSF domain at the C terminus of PEN-2 (residues 90–94) is critical for binding PEN-2 to other components in the presenilin complex and 2) the overall length of the exposed C terminus is critical for functional γ-secretase activity.

3.549 The target cell plasma membrane is a critical interface for *Salmonella* cell entry effector-host interplay

Cain, R.J., Hayward, R.D. and Koronakis, V.
Mol. Microbiol., ***54(4)***, *887-904 (2004)*

Salmonella species trigger host membrane ruffling to force their internalization into non-phagocytic intestinal epithelial cells. This requires bacterial effector protein delivery into the target cell via a type III secretion system. Six translocated effectors manipulate cellular actin dynamics, but how their direct and indirect activities are spatially and temporally co-ordinated to promote productive cytoskeletal rearrangements remains essentially unexplored. To gain further insight into this process, we applied mechanical cell fractionation and immunofluorescence microscopy to systematically investigate the subcellular localization of epitope-tagged effectors in transiently transfected and *Salmonella*-infected cultured cells. Although five effectors contain no apparent membrane-targeting domains, all six localized exclusively in the target cell plasma membrane fraction and correspondingly were visualized at the cell periphery, from where they induced distinct effects on the actin cytoskeleton. Unexpectedly, no translocated effector pool was detectable in the cell cytosol. Using parallel *in vitro* assays, we demonstrate

that the prenylated cellular GTPase Cdc42 is necessary and sufficient for membrane association of the *Salmonella* GTP exchange factor and GTPase-activating protein mimics SopE and SptP, which have no intrinsic lipid affinity. The data show that the host plasma membrane is a critical interface for effector-target interaction, and establish versatile systems to further dissect effector interplay.

3.550 Segregation of Nogo66 receptors into lipid rafts in rat brain and inhibition of Nogo66 signaling by cholesterol depletion
Yu, W., Guo, W. and Feng, L.
*FEBS Lett., **577**, 87-92 (2004)*

NogoA, a myelin-associated component, inhibits neurite outgrowth. Nogo66, a portion of NogoA, binds to Nogo66 receptor (NgR) and induces the inhibitory signaling. LINGO-1 and p75 neurotrophin receptor (p75), the low-affinity nerve growth factor receptor, are also required for NogoA signaling. However, signaling mechanisms downstream to Nogo receptor remain poorly understood. Here, we observed that NgR and p75 were colocalized in low-density membrane raft fractions derived from forebrains and cerebella as well as from cerebellar granule cells. NgR interacted with p75 in lipid rafts. In addition, disruption of lipid rafts by β-methylcyclodextrin, a cholesterol-binding reagent, reduced the Nogo66 signaling. Our results suggest an important role of lipid rafts in facilitating the interaction between NgRs and provide insight into mechanisms underlying the inhibition of neurite outgrowth by NogoA.

3.551 The behavior of peroxisomes in vitro: mammalian peroxisomes are osmotically sensitive particles
Antonenko, V.D., Sormunen, R.T. and Hiltunen, J.K.
*Am. J. Physiol., **287**, C1623-C1635 (2004)*

It has been known for a long time that mammalian peroxisomes are extremely fragile in vitro. Changes in the morphological appearance and leakage of proteins from purified particles demonstrate that peroxisomes are damaged during isolation. However, some properties of purified peroxisomes, e.g., the latency of catalase, imply that their membranes are not disrupted. In the current study, we tried to ascertain the mechanism of this unusual behavior of peroxisomes in vitro. Biochemical and morphological examination of isolated peroxisomes subjected to sonication or to freezing and thawing showed that the membrane of the particles seals after disruption, restoring permeability properties. Transient damage of the membrane leads to the formation of peroxisomal "ghosts" containing nucleoid but nearly devoid of matrix proteins. The rate of leakage of matrix proteins from broken particles depended inversely on their molecular size. The effect of polyethylene glycols on peroxisomal integrity indicated that these particles are osmotically sensitive. Peroxisomes suffered an osmotic lysis during isolation that was resistant to commonly used low-molecular-mass osmoprotectors, e.g., sucrose. Damage to peroxisomes was partially prevented by applying more "bulky" osmoprotectors, e.g., polyethylene glycol 1500. A method was developed for the isolation of highly purified and nearly intact peroxisomes from rat liver by using polyethylene glycol 1500 as an osmoprotector.

3.552 A common mechanism for the regulation of vesicular SNAREs on phospholipid membranes
Hu, K., Rickman, C., Carroll. J. and Davletov, B.
*Biochem.J., **377**, 781-765 (2004)*

The SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) family of proteins is essential for membrane fusion in intracellular traffic in eukaryotic organisms. v-SNAREs (vesicular SNAREs) must engage target SNAREs in the opposing membrane to form the fusogenic SNARE complex. Temporal and spatial control of membrane fusion is important for many aspects of cell physiology and may involve the regulation of the SNAREs resident on intracellular membranes. Here we show that the v-SNARE synaptobrevin 2, also known as VAMP (vesicle-associated membrane protein) 2, is restricted from forming the SNARE complex in chromaffin granules from adrenal medullae to the same degree as in brain-purified synaptic vesicles. Our analysis indicates that the previously reported synaptophysin–synaptobrevin interaction is not likely to be involved in regulation of the v-SNARE. Indeed, the restriction can be reproduced for two distinct v-SNARE homologues, synaptobrevin 2 and cellubrevin/VAMP3, by reconstituting them in pure liposomal membranes. Overall, our data uncover a common mechanism for the control of SNARE engagement where intact phospholipid membranes rather than proteins down-regulate vesicular SNAREs in different cellular organelles.

3.553 Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression
Neumann-Giesen, C. Et al
*Biochem. J., **378**, 509-518 (2004)*

The reggie protein family consists of two proteins, reggie-1 and -2, also called flotillins, which are highly ubiquitous and evolutionarily conserved. Both reggies have been shown to be associated with membrane rafts and are involved in various cellular processes such as T-cell activation, phagocytosis and insulin signalling. However, the exact molecular function of these proteins remains to be determined. In addition, the mechanism of membrane association of reggie-1, which does not contain any transmembrane domain, is not known. In this study, we have produced a fusion protein of reggie-1 with enhanced green fluorescent protein and generated targeted substitutions for the inactivation of putative palmitoylation and myristoylation sites. We were able to show that reggie-1 is myristoylated and multiply palmitoylated and that lipid modifications are necessary for membrane association of reggie-1. Overexpression of reggie-1 resulted in the induction of numerous filopodia-like protrusions in various cell lines, suggesting a role for reggie-1 as a signalling protein in actin-dependent processes.

3.554 Different subcellular localization of sulphotransferase 2B1b in human placenta and prostate
He, D., Meloche, C.A., Dumas, N.A., Frost, A.R. and Falany, C.N.
*Biochem. J., **379**, 533-540 (2004)*

The human hydroxysteroid SULT (sulphotransferase) 2B1 subfamily consists of two isoforms, SULT2B1a and SULT2B1b. These two isoenzymes are transcribed from the same gene by alternative splicing of their first exons and share 94% amino acid sequence identity. The SULT2B1 isoforms are highly selective for the sulphation of 3β-hydroxysteroids. Immunoblot analysis of SULT2B1 expression in several human tissues indicates the presence of only SULT2B1b protein. Immunoreactive SULT2B1b protein was detected in human prostate, skin, placenta and lung tissue. SULT2B1b mRNA expression was detected in RNA isolated from term placenta, normal prostate, prostate carcinoma, benign prostate hyperplasia, LNCaP prostate cancer cells, breast cancer specimens and MCF-7 breast cancer cells. Immunohistochemical localization of SULT2B1b, in terms placental and prostate tissues, detected it in nuclei of placental syncytiotrophoblasts and cytoplasm of epithelial cells in prostate tissues. Immunoreactive and catalytically active SULT2B1b was identified in nuclei isolated from term human placenta. Also SULT2B1b was capable of translocating to nuclei in BeWo placental cells after stable transfection and differentiation. In contrast, immunohistochemical analysis of human prostate showed only cytosolic localization of SULT2B1b in the basal and luminal prostate epithelial cells. SULT2B1b was not detected in isolated nuclei from LNCaP prostate cancer cells but was present in the cytosolic fraction. Differential subcellular localization of SULT2B1b in prostate and placenta suggests that SULT2B1b may be differentially regulated and have different physiological functions in these two hormonally responsive human tissues.

3.555 Recruitment of the cross-linked opsonic receptor CD32A (FcγRIIA) to high density detergent-resistant membrane domains in human neutrophils
Rollet-Labelle, E., Marois, S., Barbeau, K., Malawista, S.E. and Naccache, P.H.
*Biochem. J., **381**, 919-928 (2004)*

We have previously shown that CD32A (or FcγRIIA), one of the main opsonin receptors, was rapidly insolubilized and degraded in intact neutrophils after its cross-linking. In view of these experimental difficulties, the early signalling steps in response to CD32A activation were studied in purified plasma membranes of neutrophils. After CD32A cross-linking in these fractions, the tyrosine phosphorylation of two major substrates, the receptor itself and the tyrosine kinase Syk, was observed. Phosphorylation of these two proteins was observed only in the presence of orthovanadate, indicating the presence, in the membranes, of one or more tyrosine phosphatases that maintain CD32A dephosphorylation. The tyrosine phosphorylation of these two proteins was inhibited by the Src kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2). The ligation of CD32A led to its recruitment to a previously uncharacterized subset of high-density flotillin-1-positive DRMs (detergent-resistant membranes). The changes in the solubility properties of CD32A were observed in the absence of added ATP; therefore, they were probably not secondary to the tyrosine phosphorylation of the receptor, rather they preceded it. Src kinases as well as Syk were constitutively present in DRMs of high and low density and no evident changes in their distribution were detected after cross-linking of CD32A. Pretreatment of

plasma membranes with methyl-β-cyclodextrin did not inhibit the recruitment of CD32A to DRMs, although it led to the loss of the Src kinase Lyn from these fractions. In addition, methyl-β-cyclodextrin inhibited the tyrosine phosphorylation of CD32A and Syk induced by cross-linking of CD32A. This membrane model allowed us to observe a movement of CD32A from detergent-soluble regions of the membranes to DRMs, where it joined Src kinases and Syk and became tyrosine-phosphorylated.

3.556 Structure and cholesterol domain dynamics of an enriched caveolae/raft isolate
Gallegos, A.M., McIntosh, A.L., Atshaves, B.P. and Schroeder, F.
*Biochem. J., **382**, 451-461 (2004)*

Despite the importance of cholesterol in the formation and function of caveolar microdomains in plasma membranes, almost nothing is known regarding the structural properties, cholesterol dynamics or intracellular factors affecting caveolar cholesterol dynamics. A non-detergent method was employed to isolate caveolae/raft domains from purified plasma membranes of murine fibroblasts. A series of fluorescent lipid probe molecules or a fluorescent cholesterol analogue, dehydroergosterol, were then incorporated into the caveolae/raft domains to show that: (i) fluorescence polarization of the multiple probe molecules {diphenylhexatriene analogues, DiI_{18} (1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate), parinaric acids and NBD-stearic acid {12-(*N*-methyl)-*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-octadecanoic acid} indicated that acyl chains in caveolae/raft domains were significantly less 'fluid' (i.e. more rigid) and the transbilayer 'fluidity gradient' was 4.4-fold greater than in plasma membranes; (ii) although sterol was more ordered in caveolae/raft domains than plasma membranes, spontaneous sterol transfer from caveolae/raft domains was faster (initial rate, 32%; half-time, $t_{1/2}$, 57%) than from the plasma membrane; (iii) although kinetic analysis showed similar proportions of exchangeable and non-exchangeable sterol pools in caveolae/raft domains and plasma membranes, addition of SCP-2 (sterol carrier protein-2) 1.3-fold more selectively increased sterol transfer from caveolae/raft domains by decreasing the $t_{1/2}$ (50%) and increasing the initial rate (5-fold); (iv) SCP-2 was also 2-fold more selective in decreasing the amount of non-exchangeable sterol in caveolae/raft domains compared with plasma membranes, such that nearly 80% of caveolar/raft sterol became exchangeable. In summary, although caveolae/raft lipids were less fluid than those of plasma membranes, sterol domains in caveolae/rafts were more spontaneously exchangeable and more affected by SCP-2 than those of the bulk plasma membranes. Thus caveolae/raft domains isolated without the use of detergents display unique structure, cholesterol domain kinetics and responsiveness to SCP-2 as compared with the parent plasma membrane.

3.557 Rafts can trigger contact-mediated secretion of bacterial effectors via a lipid-based mechanism
Van der Goot, F.G., Tran van Nhieu, G., Allaoui, A., Sansonetti, P and Lafont, F.
*J. Biol. Chem., **279(46)**, 47792-47798 (2004)*

Infection by the Gram-negative bacterial pathogen *Shigella flexneri* depends on its ability to invade host cells. Bacterial engulfment requires a functional type III secretion system (TTSS) allowing the translocation into host cells of bacterial effectors that activate cell-signaling cascades. We demonstrated previously that specialized lipid membrane domains enriched in cholesterol and sphingolipids (rafts) are involved during early steps of invasion, namely in binding and host cell entry. In this study, we addressed the issue of contact-mediated secretion by the TTSS. We show that contact-mediated and TTSS-induced hemolysis depend on the presence of cholesterol on the host cell surface. We found that purified detergent resistant membranes were able to activate TTSS. Finally, we found that artificial liposomes, devoid of proteins, were able to activate the TTSS but only when their composition mimicked that of lipid rafts. Altogether, these data indicate that specific lipid packing can trigger contact-mediated secretion by *S. flexneri*.

3.558 Rhodopsin signaling and organization in heterozygote rhodosin knockout mice
Linag, Y. et al
*J. Biol. Chem., **279(46)**, 48189-48196 (2004)*

Rhodopsin (Rho) resides within internal membrane structures called disc membranes that are found in the rod outer segments (ROS) of photoreceptors in the retina. Rho expression is essential for formation of ROS, which are absent in knockout Rho-/- mice. ROS of mice heterozygous for the *Rho* gene deletion (Rho+/-) may have a lower Rho density than wild type (WT) membranes, or the ROS structure may be reduced in size due to lower Rho expression. Here, we present evidence that the smaller volume of ROS from heterozygous mice is most likely responsible for observed electrophysiological response differences. In

Rho+/- mice as compared with age-matched WT mice, the length of ROS was shorter by 30–40%, and the average diameter of ROS was reduced by ~20%, as demonstrated by transmission and scanning electron microscopy. Together, the reduction of the volume of ROS was ~60% in Rho+/- mice. Rho content in the eyes was reduced by ~43% and 11-*cis*-retinal content in the eye was reduced by ~38%, as determined by UV-visible spectroscopy and retinoid analysis, respectively. Transmission electron microscopy of negatively stained disc membranes from Rho+/- mice indicated a typical morphology apart from the reduced size of disc diameter. Power spectra calculated from disc membrane regions on such electron micrographs displayed a diffuse ring at ~4.5 nm^{-1}, indicating paracrystallinity of Rho. Atomic force microscopy of WT and Rho+/- disc membranes revealed, in both cases, Rho organized in paracrystalline and raftlike structures. From these data, we conclude that the differences in physiological responses measured in WT and Rho+/- mice are due to structural changes of the whole ROS and not due to a lower density of Rho.

3.559 Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains
Bravo, A. et al
*Biochim. Biophys. Acta, **1667**, 38-46 (2004)*

Bacillus thuringiensis Cry1A toxins, in contrast to other pore-forming toxins, bind two putative receptor molecules, aminopeptidase N (APN) and cadherin-like proteins. Here we show that Cry1Ab toxin binding to these two receptors depends on the toxins' oligomeric structure. Toxin monomeric structure binds to Bt-R_1, a cadherin-like protein, that induces proteolytic processing and oligomerization of the toxin (Gómez, I., Sánchez, J., Miranda, R., Bravo A., Soberón, M., FEBS Lett. (2002) 513, 242–246), while the oligomeric structure binds APN, which drives the toxin into the detergent-resistant membrane (DRM) microdomains causing pore formation. Cleavage of APN by phospholipase C prevented the location of Cry1Ab oligomer and Bt-R_1 in the DRM microdomains and also attenuates toxin insertion into membranes despite the presence of Bt-R_1. Immunoprecipitation experiments demonstrated that initial Cry1Ab toxin binding to Bt-R_1 is followed by binding to APN. Also, immunoprecipitation of Cry1Ab toxin-binding proteins using pure oligomeric or monomeric structures showed that APN was more efficiently detected in samples immunoprecipitated with the oligomeric structure, while Bt-R_1 was preferentially detected in samples immunoprecipitated with the monomeric Cry1Ab. These data agrees with the 200-fold higher apparent affinity of the oligomer than that of the monomer to an APN enriched protein extract. Our data suggest that the two receptors interact sequentially with different structural species of the toxin leading to its efficient membrane insertion.

3.560 Fas ligand is enriched in the caveolae membrane domains of thymic epithelial cells
Lalor, D., Liu, P. and Hayashi, J.
*Cellular Immunol., **230**, 10-16 (2004)*

Both Fas and Fas ligand (FasL) are expressed in the thymus. Although reports suggest that they are important throughout the thymocyte maturation process their precise role remains elusive. The present paper characterizes the expression of FasL in the thymus and in the TEA3A1 and BT1B functional thymic epithelial cell (TEC) lines. FasL expression by thymus fractions, TEA3A1, and BT1B cells was detected by Northern blot analysis. In TEA3A1 cells, we discovered that FasL protein expression was localized to caveolae membrane domains. This restricted subcellular localization of FasL, together with reports describing the localization of the major histocompatibility complex proteins, the T cell receptor and Fas to caveolae membrane domains, may provide a mechanism for the deletion of thymocytes during negative selection. Finally, using semi-quantitative RT-PCR we found that FasL expression by TECs is regulated by glucocorticoids.

3.561 Partitioning of NAP_i cotransporter in cholesterol-, sphingonyelin-, and glycophingolipid-enriched membrane domains modulates NAP_i protein diffusion, clustering, and activity
Inoue, M. et al
*J. Biol. Chem., **279(47)**, 49160-49171 (2004)*

In dietary potassium deficiency there is a decrease in the transport activity of the type IIa sodium/phosphate cotransporter protein (NaP_i) despite an increase in its apical membrane abundance. This novel posttranslational regulation of NaP_i activity is mediated by the increased glycosphingolipid content of the potassium-deficient apical membrane. However, the mechanisms by which these lipids modulate NaP_i activity have not been determined. We determined if in potassium deficiency NaP_i is increasingly

partitioned in cholesterol-, sphingomyelin-, and glycosphingolipid-enriched microdomains of the apical membrane and if the increased presence of NaP_i in these microdomains modulates its activity. By using a detergent-free density gradient flotation technique, we found that 80% of the apical membrane NaP_i partitions into the low density cholesterol-, sphingomyelin-, and GM1-enriched fractions characterized as "lipid raft" fractions. In potassium deficiency, a higher proportion of NaP_i was localized in the lipid raft fractions. By combining fluorescence correlation spectroscopy and photon counting histogram methods for control and potassium-deficient apical membranes reconstituted into giant unilamellar vesicles, we showed a 2-fold *decrease* in lateral diffusion of NaP_i protein and a greater than 2-fold *increase* in size of protein aggregates/clusters in potassium deficiency. Our results indicate that NaP_i protein is localized in membrane microdomains, that in potassium deficiency a larger proportion of NaP_i protein is present in these microdomains, and that NaP_i lateral diffusion is slowed down and NaP_i aggregation/clustering is increased in potassium deficiency, both of which could be associated with the decreased Na/P_i cotransport activity in potassium deficiency.

3.562 Nef associates with p21-activated kinase 2 in a p21-GTPase-dependent dynamic activation complex within lipid rafts

Pulkkinen, K., Renkema, G.H., Kirchhoff, F. And Saksela, K.
J. Virol., ***78(23)***, *12773-12780 (2004)*

We have previously reported that Nef specifically interacts with a small but highly active subpopulation of p21-activated kinase 2 (PAK2). Here we show that this is due to a transient association of Nef with a PAK2 activation complex within a detergent-insoluble membrane compartment containing the lipid raft marker GM1. The low abundance of this Nef-associated kinase (NAK) complex was found to be due to an autoregulatory mechanism. Although activation of PAK2 was required for assembly of the NAK complex, catalytic activity of PAK2 also promoted dissociation of this complex. Testing different constitutively active PAK2 mutants indicated that the conformation associated with p21-mediated activation rather than kinase activity per se was required for PAK2 to become NAK. Although association with PAK2 is one of the most conserved properties of Nef, we found that the ability to stimulate PAK2 activity differed markedly among divergent Nef alleles, suggesting that PAK2 association and activation are distinct functions of Nef. However, mutations introduced into the p21-binding domain of PAK2 revealed that p21-GTPases are involved in both of these Nef functions and, in addition to promoting PAK2 activation, also help to physically stabilize the NAK complex.

3.563 Identification of Epstein-Barr virus RK-BARF0-interacting proteins and characterization of expression pattern

Thornburg, N.J., Kusano, S. and Raab-Traub, N.
J. Virol., ***78(23)***, *12848-12856 (2004)*

The Epstein-Barr virus (EBV) BamHI A transcripts are a family of transcripts that are differentially spliced and can be detected in multiple EBV-associated malignancies. Several of the transcripts may encode proteins. One transcript of interest, *RK-BARF0*, is proposed to encode a 279-amino-acid protein with a possible endoplasmic reticulum-targeting sequence. In this study, the properties of RK-BARF0 were examined through identification of cellular-interacting proteins through yeast two-hybrid analysis and characterization of its expression in EBV-infected cells and tumors. In addition to the interaction previously identified with cellular Notch, it was determined that RK-BARF0 also bound cellular human I-mfa domain-containing protein (HIC), epithelin, and scramblase. An interaction between RK-BARF0 and Notch or epithelin induced proteasome-dependent degradation of Notch and epithelin but not of HIC or scramblase. Low levels of endogenous Notch expression in EBV-positive cell lines may correlate with RK-BARF0 expression. However, a screen of EBV-positive cell lines and tumors with an affinity-purified α-RK-BARF0 antiserum did not consistently detect RK-BARF0. These data suggest that while RK-BARF0 may have important cellular functions during EBV infection, and while the phenotype of EBV-positive cells suggest its expression, RK-BARF0 levels may be too low to detect.

3.564 A mammalian ortholog of *Saccharomyces cerevisiae* Vac14 that associates with and Up-regulates PIKfyve phosphoinositide 5-kinase activity

Sbrissa, D. et al
Mol. Cell Biol., ***24(23)***, *10437-10447 (2004)*

Multivesicular body morphology and size are controlled in part by PtdIns(3,5)P_2, produced in mammalian cells by PIKfyve-directed phosphorylation of PtdIns(3)P. Here we identify human Vac14 (hVac14), an evolutionarily conserved protein, present in all eukaryotes but studied principally in yeast thus far, as a novel positive regulator of PIKfyve enzymatic activity. In mammalian cells and tissues, Vac14 is a low-abundance 82-kDa protein, but its endogenous levels could be up-regulated upon ectopic expression of hVac14. PIKfyve and hVac14 largely cofractionated, populated similar intracellular locales, and physically associated. A small-interfering RNA-directed gene-silencing approach to selectively eliminate endogenous hVac14 rendered HEK293 cells susceptible to morphological alterations similar to those observed upon expression of PIKfyve mutants deficient in PtdIns(3,5)P_2 production. Largely decreased in vitro PIKfyve kinase activity and unaltered PIKfyve protein levels were detected under these conditions. Conversely, ectopic expression of hVac14 increased the intrinsic PIKfyve lipid kinase activity. Concordantly, intracellular PtdIns(3)P-to-PtdIns(3,5)P_2 conversion was perturbed by hVac14 depletion and was elevated upon ectopic expression of hVac14. These data demonstrate a major role of the PIKfyve-associated hVac14 protein in activating PIKfyve and thereby regulating PtdIns(3,5)P_2 synthesis and endomembrane homeostasis in mammalian cells.

3.565 Localization of organelle proteins by isotope tagging (LOPIT)
Dunkley, T.P.J., Watson, R., Griffin, J.L., Dupree, P. and Liley, K.S.
*Mol. Cell. Proteomics, **3**, 1128-1134 (2004)*

We describe a proteomics method for determining the subcellular localization of membrane proteins. Organelles are partially separated using centrifugation through self-generating density gradients. Proteins from each organelle co-fractionate and therefore exhibit similar distributions in the gradient. Protein distributions can be determined through a series of pair-wise comparisons of gradient fractions, using cleavable ICAT to enable relative quantitation of protein levels by MS. The localization of novel proteins is determined using multivariate data analysis techniques to match their distributions to those of proteins that are known to reside in specific organelles. Using this approach, we have simultaneously demonstrated the localization of membrane proteins in both the endoplasmic reticulum and the Golgi apparatus in *Arabidopsis*. Localization of organelle proteins by isotope tagging is a new tool for high-throughput protein localization, which is applicable to a wide range of research areas such as the study of organelle function and protein trafficking.

3.566 Modulation of cyclin D1 and early growth response factor-1 gene expression in interleukin-1β-treated rat smooth muscle cells by n-6 and n-3 polyunsaturated fatty acids
Bousserouel, S., Raymondjean, M., Brouillet, A., Bereziat, G. and Andreani, M.
*Eur. J. Biochem., **271**, 4462-4473 (2004)*

The proliferation of smooth muscle cells (SMC) is a key event in the development of atherosclerosis. In addition to growth factors or cytokines, we have shown previously that n-3 polyunsaturated fatty acids (PUFAs) act in opposition to n-6 PUFAs by modulating various steps of the inflammatory process. We have investigated the molecular mechanisms by which the incorporation of the n-6 PUFA, arachidonic acid, increases the proliferation of rat SMC treated with interleukin-1β, while the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), elicit no mitogenic response. Incorporation of EPA or DHA into SMC, which are then activated by interleukin-1β to mimic inflammation, decreases promoter activity of the cyclin D1 gene and phosphorylation of the retinoblastoma protein. Together, our data demonstrate that n-3 effects are dependent on the Ras/Raf-1/extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase pathway, and that down-regulation of the cyclin D1 promoter activity is mediated by the specific binding of the early growth response factor-1. Finally, we have shown that the incorporation of EPA and DHA also increased the concentration of caveolin-1 and caveolin-3 in caveolae, which correlated with n-3 PUFA inhibition of SMC proliferation through the mitogen-activated protein kinase pathway. We provide evidence indicating that, in contrast to n-6 PUFAs, n-3 PUFAs exert antiproliferative effects on SMC through the mitogen-activated protein kinase/ERK pathway.

3.567 Distribution of Can1p into stable domains reflects lateral protein segregation within the plasma membrane of living *S. cerevisiae* cells
Malinska, K., Malinsky, J., Opekarova, M. And Tanner, W.
*J. Cell Sci., **117**, 6031-6041 (2004)*

Recently, lipid-raft-based subdomains within the plasma membrane of living *Saccharomyces cerevisiae* cells were visualized using green fluorescent protein fusions, and non-overlapping subdomains containing either Pma1p or Can1p were distinguished. In this study, the long-term stability of the subdomains was investigated. Experiments with latrunculin A and nocodazole ruled out the involvement of cytoskeletal components in the stabilization of the subdomains. Also a putative role of the cell wall was excluded, because protoplasting of the cells changed neither the pattern nor the stability of the subdomains. By contrast, the expected inner dynamics of the membrane subdomains was documented by FRAP experiments. Finally, two other proteins were localized within the frame of the Can1p/Pma1p plasma-membrane partition. We show that Fur4p (another H^+ symporter) and Sur7p (a protein of unknown function) occupy the Can1p subdomain.

3.568 The H^+ -pyrophosphatase of *Rhodospirillum rubrun* is predominantly located in polyphosphate-rich acidocalcisomes

Seufferheld, M., Lea, C.R., Vieira, M., Oldfiled, E. and Docampo, R.
J. Biol. Chem., ***279(49)***, *51193-51202 (2004)*

Acidocalcisomes are acidic, calcium storage compartments with a H^+ pump located in their membrane that have been described in several unicellular eukaryotes, including trypanosomatid and apicomplexan parasites, algae, and slime molds, and have also been found in the bacterium *Agrobacterium tumefaciens.* In this work, we report that the H^+-pyrophosphatase (H^+-PPase) of *Rhodospirillum rubrum*, the first enzyme of this type that was identified and thought to be localized only to chromatophore membranes, is predominantly located in acidocalcisomes. The identification of the acidocalcisomes of *R. rubrum* was carried out by using transmission electron microscopy, x-ray microanalysis, and immunofluorescence microscopy. Purification of acidocalcisomes using iodixanol gradients indicated co-localization of the H^+-PPase with pyrophosphate (PP_i) and short and long chain polyphosphates (polyPs) but a lack of markers of the plasma membrane. polyP was also localized to the acidocalcisomes by using 4',6'-diamino-2-phenylindole staining and identified by using ^{31}P NMR and biochemical methods. Calcium in the acidocalcisomes increased when the bacteria were incubated at high extracellular calcium concentrations. The number of acidocalcisomes and chromatophore membranes as well as the amounts of PP_i and polyP increased when bacteria were grown in the light. Taken together, these results suggest that the H^+-PPase of *R. rubrum* has two distinct roles depending on its location acting as an intracellular proton pump in acidocalcisomes but in PP_i synthesis in the chromatophore membranes.

3.569 Bovine caveolin-2 cloning and effects of shear stress on its localization in bovine aortic endothelial cells

Boyd, N.L. et al
Endothelium, ***11****, 189-198 (2004)*

Caveolae are plasmalemmal domains enriched with cholesterol, caveolins, and signaling molecules. Normally, cells that express caveolin-1 also express caveolin-2, but this has not been demonstrated in bovine aortic endothelial cells (BAECs). Here, we show that BAECs express caveolin-2, which localizes in caveolae with caveolin-1. We have cloned the bovine caveolin-2 gene and after comparison with known protein sequences (human, murine, rat, and canine) have found divergent immunogenic regions (amino acid [aa] 21 to aa 50 and aa 79 to 88), which may explain the inability to detect caveolin-2 in different cell types. We developed a bovine caveolin-2–specific antibody to examine this protein's expression and localization in BAECs. We used differential gradient centrifugations and immunoprecipitation to show that bovine caveolin-2 and caveolin-1 form a hetero-oligomer in plasma membrane caveolae. Using immunocytochemistry we show that a pool of caveolin-2 also colocalizes with the cis-Golgi in static culture, but unlike caveolin-1, this Golgi associated pool is maintained after 1 day of shear exposure. Therefore, the interaction of caveolin-2 with caveolin-1 could play an important role in caveolae biogenesis and shear stimulated mechano-signal transduction.

3.570 Plasmin deficiency in Alzheimer's disease brains: causal or casual?

Dotti, C.G., Galvan, C. And Ledesma, M.D.
Neurodegenerative Dis., ***1****, 205-212 (2004)*

Substantial recent evidence suggests that defects in amyloid peptide degradation can be at the base of cases of sporadic Alzheimer's disease (AD). Among the discovered brain enzymes with the capacity to degrade amyloid peptide, the serine protease plasmin acquires special physiological relevance because of its low levels in areas of AD human brains with a high susceptibility to amyloid plaque accumulation. In this

article we comment on a series of observations supporting the fact that plasmin paucity in the brain is not simply a secondary event in the disease but rather a primary defect in certain cases of sporadic AD. We also refer to recent data pointing to alterations in raft membrane domains and diminished membrane cholesterol as the underlying cause. Finally, we discuss the possibility that plasmin deficiency in the brain could lead to AD symptomatology because of amyloid aggregation and the triggering of cell death signaling cascades.

3.571 **Leaky β-oxidation of a *trans* –fatty acid**
Yu, W. et al
J. Biol. Chem., ***279(50)***, *52160-52167 (2004)*

The degradation of elaidic acid (9-*trans*-octadecenoic acid), oleic acid, and stearic acid by rat mitochondria was studied to determine whether the presence of a *trans* double bond in place of a *cis* double bond or no double bond affects β-oxidation. Rat mitochondria from liver or heart effectively degraded the coenzyme A derivatives of all three fatty acids. However, with elaidoyl-CoA as a substrate, a major metabolite accumulated in the mitochondrial matrix. This metabolite was isolated and identified as 5-*trans*-tetradecenoyl-CoA. In contrast, little or none of the corresponding metabolites were detected with oleoyl-CoA or stearoyl-CoA as substrates. A kinetic study of long-chain acyl-CoA dehydrogenase (LCAD) and very long-chain acyl-CoA dehydrogenase revealed that 5-*trans*-tetradecenoyl-CoA is a poorer substrate of LCAD than is 5-*cis*-tetradecenoyl-CoA, while both unsaturated acyl-CoAs are poor substrates of very long-chain acyl-CoA dehydrogenase when compared with myristoyl-CoA. Tetradecenoic acid and tetradecenoylcarnitine were detected by gas chromatography/mass spectrometry and tandem mass spectrometry, respectively, when rat liver mitochondria were incubated with elaidoyl-CoA but not when oleoyl-CoA was the substrate. These observations support the conclusion that 5-*trans*-tetradecenoyl-CoA accumulates in the mitochondrial matrix, because it is less efficiently dehydrogenated by LCAD than is its *cis* isomer and that the accumulation of this β-oxidation intermediate facilitates its hydrolysis and conversion to 5-*trans*-tetradecenoylcarnitine thereby permitting a partially degraded fatty acid to escape from mitochondria. Analysis of this compromised but functional process provides insight into the operation of β-oxidation in intact mitochondria.

3.572 **Acidocalcisomes and the contractile vacuole complex are involved in osmoregulation in *Trypanosoma cruzi***
Rohloff, P., Montalvetti, A. and Docompo, R.
J. Biol. Chem., ***279(50)***, *52270-52281 (2004)*

Trypanosoma cruzi, the etiologic agent of Chagas disease, resists extreme fluctuations in osmolarity during its life cycle. *T. cruzi* possesses a robust regulatory volume decrease mechanism that completely reverses cell swelling when submitted to hypo-osmotic stress. The efflux of amino acids and K^+ release could account for only part for this volume reversal. In this work we demonstrate that swelling of acidocalcisomes mediated by an aquaporin and microtubule- and cyclic AMP-mediated fusion of acidocalcisomes to the contractile vacuole complex with translocation of this aquaporin and the resulting water movement are responsible for the volume reversal not accounted for by efflux of osmolytes. Contractile vacuole bladders were isolated by subcellular fractionation in **iodixanol** gradients, showed a high concentration of basic amino acids and inorganic phosphate, and were able to transport protons in the presence of ATP or pyrophosphate. Taken together, these results strongly support a role for acidocalcisomes and the contractile vacuole complex in osmoregulation and identify a functional role for aquaporin in protozoal osmoregulation.

3.573 **Intracellular processing and activation of membrane type 1 matrix metalloprotease depends on its partitioning into lipid domains**
Mazzone, M. et al
J. Cell Sci., ***117***, *6275-6287 (2004)*

The integral membrane type 1 matrix metalloprotease (MT1-MMP) is a pivotal protease in a number of physiological and pathological processes and confers both non-tumorigenic and tumorigenic cell lines with a specific growth advantage in a three-dimensional matrix. Here we show that, in a melanoma cell line, the majority (80%) of MT1-MMP is sorted to detergent-resistant membrane fractions; however, it is only the detergent-soluble fraction (20%) of MT1-MMP that undergoes intracellular processing to the mature form. Also, this processed MT1-MMP is the sole form responsible for ECM degradation in vitro. Finally, furin-

dependent processing of MT1-MMP is shown to occur intracellularly after exit from the Golgi apparatus and prior to its arrival at the plasma membrane. It is thus proposed that the association of MT1-MMP with different membrane subdomains might be crucial in the control of its different activities: for instance in cell migration and invasion and other less defined ones such as MT1-MMP-dependent signaling pathways.

3.574 Role of myosin VIIa and Rab27a in the motility and localization of RPE nelanosomes
Gibbs, D. Et al
*J. Cell Sci., **117**, 6473-6483 (2004)*

Myosin VIIa functions in the outer retina, and loss of this function causes human blindness in Usher syndrome type 1B (USH1B). In mice with mutant *Myo7a*, melanosomes in the retinal pigmented epithelium (RPE) are distributed abnormally. In this investigation we detected many proteins in RPE cells that could potentially participate in melanosome transport, but of those tested, only myosin VIIa and Rab27a were found to be required for normal distribution. Two other expressed proteins, melanophilin and myosin Va, both of which are required for normal melanosome distribution in melanocytes, were not required in RPE, despite the association of myosin Va with the RPE melanosome fraction. Both myosin VIIa and myosin Va were immunodetected broadly in sections of the RPE, overlapping with a region of apical filamentous actin. Some 70-80% of the myosin VIIa in RPE cells was detected on melanosome membranes by both subcellular fractionation of RPE cells and quantitative immunoelectron microscopy, consistent with a role for myosin VIIa in melanosome motility. Time-lapse microscopy of melanosomes in primary cultures of mouse RPE cells demonstrated that the melanosomes move in a saltatory manner, interrupting slow movements with short bursts of rapid movement (>1 μm/second). In RPE cells from *Myo7a*-null mice, both the slow and rapid movements still occurred, except that more melanosomes underwent rapid movements, and each movement extended approximately five times longer (and further). Hence, our studies demonstrate the presence of many potential effectors of melanosome motility and localization in the RPE, with a specific requirement for Rab27a and myosin VIIa, which function by transporting and constraining melanosomes within a region of filamentous actin. The presence of two distinct melanosome velocities in both control and *Myo7a*-null RPE cells suggests the involvement of at least two motors other than myosin VIIa in melanosome motility, most probably, a microtubule motor and myosin Va.

3.575 N-octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease
Lin, H. et al
*Biochim. Biophys. Acta, **1689**, 219-228 (2004)*

Gaucher disease (GD) is the most common form of sphingolipidosis and is caused by a defect of β-glucosidase (β-Glu). A carbohydrate mimic *N*-octyl-β-valienamine (NOV) is an inhibitor of β-Glu. When applied to cultured GD fibroblasts with F213I β-Glu mutation, NOV increased the protein level of the mutant enzyme and up-regulated cellular enzyme activity. The maximum effect of NOV was observed in F213I homozygous cells in which NOV treatment at 30 μM for 4 days caused a ~6-fold increase in the enzyme activity, up to ~80% of the activity in control cells. NOV was not effective in cells with other β-Glu mutations, N370S, L444P, 84CG and RecNciI. Immunofluorescence and cell fractionation showed localization of the F213I mutant enzyme in the lysosomes of NOV-treated cells. Consistent with this, NOV restored clearance of ^{14}C-labeled glucosylceramide in F213I homozygous cells. F213I mutant β-Glu rapidly lost its activity at neutral pH in vitro and this pH-dependent loss of activity was attenuated by NOV. These results suggest that NOV works as a chemical chaperone to accelerate transport and maturation of F213I mutant β-Glu and may suggest a therapeutic value of this compound for GD.

3.576 Overexpression of caveolin-1 increases plasma membrane fluidity and reduces P-glycoprotein function in Hs578T/Dox
Cai, C., Zhu, H. and Chen, J.
*Biochem. Biophys Res. Comm., **320**, 868-874 (2004)*

Cholesterol is a key lipid in mediating the enzyme activity or signaling pathway of many proteins on the plasma membrane in mammalian cells. In this report, we demonstrate for the first time that after overexpressing caveolin-1, the plasma membrane cholesterol level was decreased by about 12% and 30% for doxorubicin-sensitive and doxorubicin-resistant Hs578T breast cancer cells, respectively. However, the total cholesterol level in both cell lines was increased by about 10%. By measuring fluorescence and flow cytometry using the fluorescence dyes 1,6-diphenyl-1,3,5-hexatriene and Merocyanine 540, we found that

overexpressing caveolin-1 resulted in a similar increase in membrane fluidity and loosening of lipid packing density as cholesterol depletion by 1 mM methyl-β-cyclodextrin (MβCD) or 2-hydroxypropyl-β-cyclodextrin (HβCD). Moreover, we found that the transport activity of P-gp was significantly inhibited by 1 mM MβCD or HβCD, which is also similar to the inhibitory effect of caveolin-1 overexpression. Our data demonstrate for the first time that the reduction of the plasma membrane cholesterol level induced by overexpressing caveolin-1 may indirectly inhibit P-gp transport activity by increasing plasma membrane fluidity.

3.577 Depression of transcription and translation during daily torpor in the Djungarian hamster (*Phodopus sungorus)*
Diaz, M.B., Lange, M., Heldmaier, G. And Klingenspor, M.-
*J. Comp. Physiol. B, **174**, 495-502 (2004)*

During daily torpor, Djungarian hamsters reduce their metabolic rate by more than 70% below their resting metabolic rate for several hours per day. We investigated whether this depression of metabolism is associated with a reduction in transcription and translation. Liver tissue was sampled in defined metabolic states: during normometabolism, in the torpid state and after arousal from torpor. Nuclei were isolated from liver tissue and subjected to nuclear run-on assays at an assay temperature of 25 °C. We observed a ~40% decrease in transcriptional initiation in liver nuclei of hamsters which had attained minimal metabolic rate during torpor as compared to nuclei from normometabolic hamsters. During arousal from torpor, the transcriptional run-on activity recovered to the normometabolic level. Polysome profile analysis of liver tissue was used to determine the proportion of actively translating polysomes. Profiles of liver samples from torpid animals show a disaggregation of polysomes compared to profiles from normometabolic hamsters, which indicates that, in addition to transcription, protein synthesis decreases during torpor. These results indicate that during torpor a specific inhibition of the energetically costly processes of RNA and protein synthesis contributes to the overall metabolic depression.

3.578 Tyrosine phosphatase activity in mitochondria: presence of Shp-2 phosphatase in mitochondria
Salvi, M. Et al
*Cell. Mol. Life Sci., **61**, 2393-2404 (2004)*

Tyrosine phosphorylation by unidentified enzymes has been observed in mitochondria, with recent evidence indicating that non-receptorial tyrosine kinases belonging to the Src family, which represent key players in several transduction pathways, are constitutively present in mitochondria. The extent of protein phosphorylation reflects a coordination balance between the activities of specific kinases and phophatases. The present study demonstrates that purified rat brain mitochondria possess endogenous tyrosine phosphatase activity. Mitochondrial phosphatases were found to be capable of dephosphorylating different exogenous substrates, including paranitrophenylphosphate, ^{32}P-poly(Glu-Tyr)$_{4:1}$ and ^{32}P-angiotensin. These activities are strongly inhibited by peroxovanadate, a well-known inhibitor of tyrosine phosphatases, but not by inhibitors of alkali or Ser/Thr phosphatases, and mainly take place in the intermembrane space and outer mitochondrial membrane. Using a combination of approaches, we identified the tyrosine phosphatase Shp-2 in mitochondria. Shp-2 plays a crucial role in a number of intracellular signalling cascades and is probably involved in several human diseases. It thus represents the first tyrosine phosphatase shown to be present in mitochondria.

3.579 Paradoxical downregulation of the glucose oxidation pathway despite enhanced flux in severe heart failure
Lei, B. Et al
*J. Mol. Cell. Cardiol., **36**, 567-576 (2004)*

Free fatty acid (FFA) oxidation is depressed in severe heart failure due to reduced activity of mitochondrial fatty acid oxidation enzymes. It is unknown whether the concomitant enhancement in cardiac glucose use is a consequence of reduced FFA oxidation, or also due to potentiation of the carbohydrate oxidative pathway. FFA and glucose oxidation rates were measured *in vivo* in 9 normal dogs and 9 dogs with pacing-induced heart failure by infusing ^{3}H-oleate and ^{14}C-glucose. FFA oxidation was lower (39 ± 9 vs. 73 ± 5 nmol min^{-1} g^{-1}), while glucose oxidation was higher (42 ± 8 vs. 17 ± 6 nmol min^{-1} g^{-1}) in failing compared to normal hearts ($P < 0.05$). At the end of the *in vivo* experiment, clamp-frozen biopsies were harvested from the left ventricle. Messenger RNAs encoding for proteins involved in both glucose and fatty acid metabolism, and for citrate synthase, were significantly reduced. Protein expression of GLUT-1 and GLUT-4, and GLUT-4 translocation to the sarcolemma showed no significant differences between the

two groups despite a significant reduction in mRNAs with heart failure. GAPDH mRNA, protein expression, and activity were all reduced. The E2 subunit of pyruvate dehydrogenase was decreased both at the mRNA and protein level, with no effect on either fractional or maximal activity. In conclusion, we found either no changes or moderate downregulation of key enzymes of the carbohydrate metabolism in failing hearts, which suggests that the increase in glucose oxidation *in vivo* was principally due to impaired FFA oxidation and that the maximal myocardial capacity to obtain energy from substrate is globally depressed.

3.580 Acidocalsisomes of *Phytomonas francai* possess distinct morphological characteristics and contain iron

Miranda, K. et al
*Microsc. Microanal., **10**, 647-655 (2004)*

Acidocalcisomes are acidic calcium storage compartments described initially in trypanosomatid and apicomplexan parasites, and recently found in other unicellular eukaryotes. The aim of this study was to identify the presence of acidocalcisomes in the plant trypanosomatid Phytomonas francai. Electron-dense organelles of P. francai were shown to contain large amounts of oxygen, sodium, magnesium, phosphorus, potassium, calcium, iron, and zinc as determined by X-ray microanalysis, either in situ or when purified using iodixanol gradient centrifugation or by elemental mapping. The presence of iron is not common in other acidocalcisomes. In situ, but not when purified, these organelles showed an elongated shape differing from previously described acidocalcisomes. However, these organelles also possessed a vacuolar H+-pyrophosphatase (V-H+-PPase) as determined by biochemical methods and by immunofluorescence microscopy using antibodies against the enzyme. Together, these results suggest that the electron-dense organelles of P. francai are homologous to the acidocalcisomes described in other trypanosomatids, although with distinct morphology and elemental content.

3.581 Mechanisms of neurotrophin receptor vesicular transport

Yano, H. and Chao, M.V.
*J. Neurobiol**., **58**, 244-257 (2004)*

Accumulating evidence has indicated that neurotrophin receptor trafficking plays an important role in neurotrophin-mediated signaling in developing as well as mature neurons. However, little is known about the molecular mechanisms and the components of neurotrophin receptor vesicular transport. This article will describe how neurotrophin receptors, Trk and p75 neurotrophin receptor ($p75^{NTR}$), are intimately involved in the axonal transport process. In particular, the molecules that may direct Trk receptor trafficking in the axon will be discussed. Finally, potential mechanisms by which receptor-containing vesicles link to molecular cytoskeletal motors will be presented.

3.582 Effect of Membrane Perturbants on the Activity and Phase Distribution of Inositol Phosphorylceramide Synthase; Development of a Novel Assay

Aeed, P.A., Sperry, A.E., Young, C.L., Nagiec, M.M. andf Elhammer, Å.P.
*Biochemistry, **43**, 8483-8493 (2004)*

The effect of 26 different membrane-perturbing agents on the activity and phase distribution of inositol phosphorylceramide synthase (IPC synthase) activity in crude *Candida albicans* membranes was investigated. The nonionic detergents Triton X-100, Nonidet P-40, Brij, Tween, and octylglucoside all inactivated the enzyme. However, at moderate concentrations, the activity of the Triton X-100- and octylglucoside-solubilized material could be partially restored by inclusion of 5 mM phosphatidylinositol (PI) in the solubilization buffer. The apparent molecular mass of IPC synthase activity solubilized in 2% Triton X-100 was between 1.5×10^6 and 20×10^6 Da, while under identical conditions, octylglucoside-solubilized activity remained associated with large presumably membrane-like structures. Increased detergent concentrations produced more drastic losses of enzymatic activity. The zwitterionic detergents Empigen BB, *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB), Zwittergent 3-10, and amidosulfobetaine (ASB)-16 all appeared capable of solubilizing IPC synthase. However, these agents also inactivated the enzyme essentially irreversibly. Solubilization with lysophospholipids again resulted in drastic losses of enzymatic activity that were not restored by the inclusion of PI. Lysophosphatidylinositol also appeared to compete, to some extent, with the donor substrate phosphatidylinositol. The sterol-containing agent digitonin completely inactivated IPC synthase. By contrast, sterol-based detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), and taurodeoxycholate

(tDOC) had little or no effect on the enzyme activity. The IPC synthase activity in *C. albicans* membranes remained largely intact and sedimentable at CHAPS concentrations (4%) where >90% of the phospholipids and 60% of the total proteins were extracted from the membranes. At 2.5% CHAPS, a concentration where approximately 50% of the protein and 80% of the phospholipids are solubilized, there was no detectable loss of enzyme activity, and it was found that the detergent-treated membranes had significantly improved properties compared to crude, untreated membranes as the source of IPC synthase activity. In contrast to assays utilizing intact membranes or Triton X-100 extracts, assays using CHAPS- or tDOC-washed membranes were found to be reproducible, completely dependent on added acceptor substrate (C_6-7-nitro-2-1,3-benzoxadiazol-4-yl (NBD)-ceramide), and >95% dependent on added donor substrate (PI). Product formation was linear with respect to both enzyme concentration and time, and transfer efficiency was improved more than 20-fold as compared to assays using crude membranes. Determination of kinetic parameters for the two IPC synthase substrates using CHAPS-washed membranes resulted in K_m values of 3.3 and 138.0 μM for C_6-NBD-ceramide and PI, respectively. In addition, the donor substrate, PI, was found to be inhibitory at high concentrations with an apparent K_i of 588.2 μM.

3.583

3.584 CD4-induced down-regulation of T cell adhesion to B cells is associated with localization of phosphatidyl inositol 3-kinase and LFA-1 in distinct membrane domains
Trucy, M., Barbat, C., Sorice, M., Fischer, A. and Mazerolles, F.
*Eur. J: Immunol., **34(8)**, 2168-2178 (2004)*

We have previously shown that binding of anti-CD4 antibody inhibit LFA-1-dependent adhesion between $CD4^+$ T cells and B cells in a $p56^{lck}$ and a PI3-kinase-dependent manner. In this work, we investigated with two different T cell lines (Jurkat and A201) whether CD4 binding could alter interactions of the proteins putatively involved in this adhesion regulatory pathway. Anti-CD4 binding was shown to induce a transient association between PI3-kinase and LFA-1, which took place in different regions of the plasma membrane. It was detected in detergent soluble membrane but also in detergent insoluble membrane consisting in raft microdomains, composed of GM1 and/or GM3 gangliosides. These results show that anti-CD4 Ab could modify the interaction between LFA-1 and signaling molecules, such as PI3-kinase and induce, in part, their recruitment in raft domains. By using specific inhibitors, raft integrity and CD4 association with GM3 were found necessary for observing the CD4-dependent inhibition of LFA-1-mediated adhesion. These results strongly suggest that these molecular rearrangements in the membrane are necessary to induce down-regulation of LFA-1-mediated adhesion.

3.585 Comparison of nerve terminal events in vivo effecting retrograde transport of vesicles containing neurotrophins or synaptic vesicle components
Weible II, M.W., Ozsarac, N., Grimes, M.L. and Hendry, I.A.
*J. Neurosci. Res., **75(6)**, 771-781 (2004)*

Although vesicular retrograde transport of neurotrophins in vivo is well established, relatively little is known about the mechanisms that underlie vesicle endocytosis and formation before transport. We demonstrate that in vivo not all retrograde transport vesicles are alike, nor are they all formed using identical mechanisms. As characterized by density, there are at least two populations of vesicles present in the synaptic terminal that are retrogradely transported along the axon: those containing neurotrophins (NTs) and those resulting from synaptic vesicle recycling. Vesicles containing nerve growth factor (NGF), NT-3, or NT-4 had similar densities with peak values at about 1.05 g/ml. Synaptic-derived vesicles, labeled with anti-dopamine β-hydroxylase (DBH), had densities with peak values at about 1.16 g/ml. We assayed the effects of pharmacologic agents in vivo on retrograde transport from the anterior eye chamber to the superior cervical ganglion. Inhibitors of phosphatidylinositol-3-OH (PI-3) kinase and actin function blocked transport of both anti-DBH and NGF, demonstrating an essential role for these molecules in retrograde transport of both vesicle types. Dynamin, a key element in synaptic vesicle recycling, was axonally transported in retrograde and anterograde directions, and compounds able to interfere with dynamin function had a differential effect on retrograde transport of NTs and anti-DBH. Okadaic acid significantly decreased retrograde axonal transport of anti-DBH and increased NGF retrograde transport. We conclude that there are both different and common proteins involved in endocytosis and targeting of retrograde transport of these two populations of vesicles.

3.586 Distinct protective mechanisms of HO-1 and HO-2 against hydroperoxide-induced cytotoxicity
Kim, Y-S., Zhuang, H., Koehler, R.C. and Dore, S.
*Free Radical Biology & Medicine, **38**, 85-92 (2005)*

Heme oxygenases (HO-1 and HO-2) catalyze the NADPH-cytochrome P_{450} reductase (CPR)-dependent degradation of heme into iron, carbon monoxide, and biliverdin, which is reduced into bilirubin. Under basal conditions, HO-1 is often undetected and can be induced by numerous stress conditions. Although HO-2 is constitutively expressed, its activity appears to be regulated by post-translational modifications. HO activity has been associated with cellular protection, by which it degrades heme, a prooxidant, into bioactive metabolites. Under given circumstances, overexpression of HO-1 can render cells more sensitive to free radicals. Here, we investigated the properties of human HO isoforms that protect against oxidative stress. Considering that CPR can be a limiting factor for optimal HO activity, we tested stable HO-1 and HO-2 cell lines that derived from the CPR cells. Results indicate that the HO-1 and HO-2 cells are more resistant than controls to hemin and to the organic *tert*-butyl hydroperoxide, *t*-BuOOH. However, HO-1 cells are less resistant than HO-2 cells to hydrogen peroxide (H_2O_2). The levels of oxidatively modified proteins of HO-1 and HO-2 cells in response to *t*-BuOOH toxicity are identical, but the level of oxidatively modified proteins of HO-2 cells is less than that of HO-1 cells in response to H_2O_2 toxicity. Performing subcellular fractionations revealed that HO-2 and CPR are found together in the microsomal fractions, whereas HO-1 is partially present in the microsome and also found in other fractions, such as the cytosol. These same findings were observed in non-transfected primary neurons where HO-1 proteins were chemically induced with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$). The differences in subcellular localization of HO-1 and HO-2 could explain some of the discrepancies in their cellular activity and enzymatic protective mechanisms.

3.587 Calpain and other cytosolic proteases can contribute to the degradation of retro-translocated prion protein in the cytosol

Wang, X., Wang, F., Sy, M-S- and Ma, J.
J. Biol. Chem., ***280(1)***, *317-325 (2005)*

PrP, a cell surface-localized *N*-linked glycoprotein, is required for the pathogenesis of prion diseases. Recent studies have revealed that prion protein (PrP) becomes neurotoxic and prone to aggregation when it is in the cytosol, suggesting that cytosolic PrP may play a role in the pathogenesis of prion disease. Retro-translocation of PrP from the endoplasmic reticulum to the cytosol for proteasome degradation offers a natural route for PrP to enter the cytosol, but whether PrP is subject to retrotranslocation is controversial. In this study, we investigated the metabolism of endogenous wild-type PrP in several cell lines and in primary mouse cortical neurons. Our results suggest that a portion of the endogenous wild-type PrP is retro-translocated to the cytosol and degraded by the proteasome. Moreover, we also found that calpain and other cytosolic proteases could degrade PrP in the cytosol when the proteasome activity is compromised. These results provide the foundation for the hypothesis that cytosolic PrP may be involved in the pathogenesis of prion disease.

3.588 Exogenous antigens are processed through the endoplasmic reticulum-associated degradatin (ERAD) in cross-presentation by dendritic cells

Imai, J., Hasegawa, H., Maruya, M., Koyasu, S. and Yahara, I.
Int. Immunol., ***17(1)***, *45-53 (2005)*

Antigen cross-presentation is critical in infectious and tumor immunity where cytotoxic T lymphocytes are induced by dendritic cells specifically equipped with cellular machineries to present exogenous antigens with major histocompatibility complex (MHC) class I molecules. To examine molecular mechanisms of antigen cross-presentation, we employed as a model system a murine dendritic cell line DC2.4 capable of presenting soluble antigens such as ovalbumin (OVA) with MHC class I. Here, we demonstrate that exogenously added OVA is accumulated in the endoplasmic reticulum (ER) and late endosomes followed by retrograde transport to the cytoplasm through the Sec61 transporter complexes, and that CHIP functions as an E3 ubiquitin–ligase for OVA degradation by proteasomes. This mechanism is essentially the same as that known as the ER-associated degradation (ERAD) in the quality control of secretary and membrane proteins.

3.589 Distinct localization of lipid rafts and externalized phosphotidylserine at the surface of apoptotic cells

Ishii, H. et al
Biochem. Biophys. Res. Comm., ***327***, *94-99 (2005)*

Externalization of phosphatidylserine (PS) takes place in apoptotic cells as well as in viable cells under certain circumstances. Recent studies showed that externalized PS is localized at the lipid raft in viable activated immune cells. We found that lipid rafts and PS existed in a mutually exclusive manner in apoptotic cells. The number of PS-exposing apoptotic cells decreased when lipid rafts were disrupted. BCθ, which binds selectively to cholesterol in a cholesterol-rich region, did not effectively recognize lipid rafts of apoptotic cells. Lipid rafts rich in GM1 were successfully prepared from apoptotic cells, but the lipid raft protein LAT was not enriched in the preparation. Furthermore, the amount of PS and phosphatidylethanolamine but not of cholesterol in lipid rafts appeared to change after induction of apoptosis. These results suggest that lipid rafts are structurally modified during apoptosis and, despite being localized differently from PS, are involved in the externalization of PS.

3.590 FAT/CD36-mediated long-chain fatty acid uptake in adipocytes requires plasma membrane rafts
Pohl, J., Ring, A., Korkmaz, U., Ehehalt, R and Stremmel, W.
*Mol. Biol. Cell, **16(1)**, 24-31 (2005)*

We previously reported that lipid rafts are involved in long-chain fatty acid (LCFA) uptake in 3T3-L1 adipocytes. The present data show that LCFA uptake does not depend on caveolae endocytosis because expression of a dominant negative mutant of dynamin had no effect on uptake of [^{3}H]oleic acid, whereas it effectively prevented endocytosis of cholera toxin. Isolation of detergent-resistant membranes (DRMs) from 3T3-L1 cell homogenates revealed that FAT/CD36 was expressed in both DRMs and detergent-soluble membranes (DSMs), whereas FATP1 and FATP4 were present only in DSMs but not DRMs. Disruption of lipid rafts by cyclodextrin and specific inhibition of FAT/CD36 by sulfo-*N*-succinimidyl oleate (SSO) significantly decreased uptake of [^{3}H]oleic acid, but simultaneous treatment had no additional or synergistic effects, suggesting that both treatments target the same mechanism. Indeed, subcellular fractionation demonstrated that plasma membrane fatty acid translocase (FAT/CD36) is exclusively located in lipid rafts, whereas intracellular FAT/CD36 cofractionated with DSMs. Binding assays confirmed that [^{3}H]SSO predominantly binds to FAT/CD36 within plasma membrane DRMs. In conclusion, our data strongly suggest that FAT/CD36 mediates raft-dependent LCFA uptake. Plasma membrane lipid rafts might control LCFA uptake by regulating surface availability of FAT/CD36.

3.591 Ypt3132 GTPases and their novel F-Box effector protein Rcy1 regulate protein recycling
Chen, S.H. et al
*Mol. Biol. Cell, **16(1)**, 178-192 (2005)*

Ypt/Rab GTPases control various aspects of vesicle formation and targeting via their diverse effectors. We report a new role for these GTPases in protein recycling through a novel effector. The F-box protein Rcy1, which mediates plasma membrane recycling, is identified here as a downstream effector of the Ypt31/32 GTPase pair because it binds active GTP-bound Ypt31/32 and colocalizes with these GTPases on late Golgi and endosomes. Furthermore, Ypt31/32 regulates the polarized localization and half-life of Rcy1. This suggests that Ypt/Rabs can regulate the protein level of their effectors, in addition to the established ways by which they control their effectors. We show that like Rcy1, Ypt31/32 regulate the coupled phosphorylation and recycling of the plasma membrane v-SNARE Snc1. Moreover, Ypt31/32 and Rcy1 regulate the recycling of the furin-homolog Kex2 to the Golgi. Therefore, Ypt31/32 and Rcy1 mediate endosome-to-Golgi transport, because this is the only step shared by Snc1 and Kex2. Finally, we show that Rcy1 physically interacts with Snc1. Based on this result and because F-box proteins serve as adaptors between specific substrates and ubiquitin ligases, we propose that Ypt31/32 GTPases regulate the function of Rcy1 in the phosphorylation and/or ubiquitination of proteins that recycle through the Golgi.

3.592 Trafficking and localization of platinum complexes in cisplatin-resistant cell lines monitored by fluorescence-labeled platinum
Liang, X-J. et al
*J. Cell. Physiol., **202**, 635-641 (2005)*

Cisplatin is a chemotherapeutic agent commonly used in the treatment of a wide variety of malignant tumors. Resistance to cisplatin represents a major obstacle to effective cancer therapy because clinically significant levels of resistance quickly emerge after treatment. Based on previous studies indicating abnormal plasma membrane protein trafficking in cisplatin-resistant (CP-r) cells, Fluorescence (Alexa Fluor)-labeled cisplatin was used to determine whether this defect altered the trafficking and localization

of cisplatin by comparing drug sensitive KB-3-1 and KB-CP-r cells. Alexa Fluor-cisplatin was readily internalized and localized throughout the KB-3-1 cells, but overall fluorescence decreased in KB-CP-r cells, as detected by flow cytometry (FACS) and confocal microscopy. Only punctate cytoplasmic staining was observed in KB-CP-r cells with less fluorescence observed in the nucleus. Colocalization experiments with a Golgi-selective stain indicate the involvement of Golgi-like vesicles in initial intracellular processing of Alexa Fluor conjugated cisplatin complexes. As detected using an antibody to Alexa Fluor-cisplatin, cisplatin complex-binding proteins (CCBPs) were reduced in membrane fractions of single-step cisplatin-resistant KB-CP.5 cells, and increased in the cytoplasm of KB-CP.5 cells compared to KB-3-1 cells. CCBPs localized to lower density fractions in KB-CP.5 cells than in KB-3-1 cells as determined by iodixanol gradient centrifugation. In summary, inappropriate trafficking of CCBPs might explain resistance to cisplatin in cultured cancer cells, presumably because membrane binding proteins for cisplatin are not properly located on the cell surface in these cells, but are instead trapped in low density vesicles within the cytoplasm.

3.593 Fragile X protein functions with LgI and the PAR complex in flies and mice
Zarnescu, D.C. et al
*Developmental Cell, **8**, 43-52 (2005)*

Fragile X syndrome, the most common form of inherited mental retardation, is caused by loss of function for the *Fragile X Mental Retardation 1* gene (*FMR1*). FMR1 protein (FMRP) has specific mRNA targets and is thought to be involved in their transport to subsynaptic sites as well as translation regulation. We report a saturating genetic screen of the *Drosophila* autosomal genome to identify functional partners of *dFmr1*. We recovered 19 mutations in the tumor suppressor *lethal (2) giant larvae* (*dlgl*) gene and 90 mutations at other loci. *dlgl* encodes a cytoskeletal protein involved in cellular polarity and cytoplasmic transport and is regulated by the PAR complex through phosphorylation. We provide direct evidence for a Fmrp/Lgl/mRNA complex, which functions in neural development in flies and is developmentally regulated in mice. Our data suggest that Lgl may regulate Fmrp/mRNA sorting, transport, and anchoring via the PAR complex.

3.594 Kaposi's sarcoma-associated herpesvirus modulates microtubele dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus
Naranatt, P.P., Krishnan, H.H., Smith, M.S. and Chandran, B.
*J. Virol., **79(2)**, 1191-1206 (2005)*

Human herpesvirus 8 (HHV-8; also called Kaposi's sarcoma-associated herpesvirus), which is implicated in the pathogenesis of Kaposi's sarcoma (KS) and lymphoproliferative disorders, infects a variety of target cells both in vivo and in vitro. HHV-8 binds to several in vitro target cells via cell surface heparan sulfate and utilizes the α3ß1 integrin as one of its entry receptors. Interactions with cell surface molecules induce the activation of host cell signaling cascades and cytoskeletal changes (P. P. Naranatt, S. M. Akula, C. A. Zien, H. H. Krishnan, and B. Chandran, J. Virol. **77:**1524-1539, 2003). However, the mechanism by which the HHV-8-induced signaling pathway facilitates the complex events associated with the internalization and nuclear trafficking of internalized viral DNA is as yet undefined. Here we examined the role of HHV-8-induced cytoskeletal dynamics in the infectious process and their interlinkage with signaling pathways. The depolymerization of microtubules did not affect HHV-8 binding and internalization, but it inhibited the nuclear delivery of viral DNA and infection. In contrast, the depolymerization of actin microfilaments did not have any effect on virus binding, entry, nuclear delivery, or infection. Early during infection, HHV-8 induced the acetylation of microtubules and the activation of the RhoA and Rac1 GTPases. The inactivation of Rho GTPases by *Clostridium difficile* toxin B significantly reduced microtubular acetylation and the delivery of viral DNA to the nucleus. In contrast, the activation of Rho GTPases by *Escherichia coli* cytotoxic necrotizing factor significantly augmented the nuclear delivery of viral DNA. Among the Rho GTPase-induced downstream effector molecules known to stabilize the microtubules, the activation of RhoA-GTP-dependent diaphanous 2 was observed, with no significant activation in the Rac- and Cdc42-dependent PAK1/2 and stathmin molecules. The nuclear delivery of viral DNA increased in cells expressing a constitutively active RhoA mutant and decreased in cells expressing a dominant-negative mutant of RhoA. HHV-8 capsids colocalized with the microtubules, as observed by confocal microscopic examination, and the colocalization was abolished by the destabilization of microtubules with nocodazole and by the phosphatidylinositol 3-kinase inhibitor affecting the Rho GTPases. These results suggest that HHV-8 induces Rho GTPases, and in doing so, modulates microtubules and promotes the trafficking of viral capsids and the establishment of infection. This is the first demonstration of virus-induced host cell signaling pathways in the modulation of microtubule dynamics and in the trafficking of viral DNA to the

infected cell nucleus. These results further support our hypothesis that HHV-8 manipulates the host cell signaling pathway to create an appropriate intracellular environment that is conducive to the establishment of a successful infection.

3.595 Analyses of murine *Postsynaptic density-95* identify novel isoforms and potential translational control elements

Bence, M., Arbuckle, M.I., Dickson, K.S. and Grant, S.G.N.
*Mol. Brain res., **133(1)**, 143-152 (2005)*

Postsynaptic density-95 (PSD-95) is an evolutionarily conserved synaptic adaptor protein that is known to bind many proteins including the NMDA receptor. This observation has implicated it in many NMDA receptor-dependent processes including spatial learning and synaptic plasticity. We have cloned and characterised the murine *PSD-95* gene. In addition, we have identified two previously uncharacterised splice variants of the major murine PSD-95 transcript (*PSD-95α*): *PSD-95α-2b* results from an extension of exon 2 and *PSD-95α-Δ18* from the temporal exclusion of exon 18. The presence of PSD-95α-2b sequences in other PSD-95 family members implicates this peptide stretch as functionally significant. Another potential transcript (*PSD-95γ*) was also identified based on examination of EST databases. Immunoprecipitation assays demonstrate that proteins corresponding in size to PSD-95α-Δ18 and PSD-95γ interact with the NMDA receptor, suggesting an important biological role for these isoforms. Finally, we have performed bioinformatics analyses of the PSD-95 mRNA untranslated regions, identifying multiple translational control elements that suggest protein production could be regulated post-transcriptionally. The variety of mRNA isoforms and regulatory elements identified provides for a high degree of diversity in the structure and function of PSD-95 proteins.

3.596 Isoform-specific regulation of adenylyl cylase function by disruption of membrane trafficking

Ding, Q., Gros, R., Chorazyczewski, J., Ferguson, S.S.g. and Feldman, R.D.
*Mol. Pharmacol., **6782)**, 564-571 (2005)*

Oligomerization plays an important role in endoplasmic reticulum processing and membrane insertion (and ultimately in regulation of function) of a number of transmembrane spanning proteins. Furthermore, it is known that adenylyl cyclases (ACs), critical regulators of cellular functions, associate into higher order (dimeric) forms. However, the importance of these higher order aggregates in regulating adenylyl cyclase activity or trafficking to the cell membrane is unclear. Therefore, we examined the potential role of oligomerization in the membrane trafficking of adenylyl cyclase. For this purpose, the ability of full-length adenylyl cyclase and various truncation mutants to self-assemble and to be targeted to the cell membrane was assessed. A truncation mutant comprised of the initial six transmembrane spanning domains and half of the C1 catalytic domain coimmunoprecipitated with full-length AC VI. Using both biotinylation assays and assessment of enzyme distribution using sucrose density gradients, we demonstrate that expression of this mutant in human embryonic kidney 293 cells impaired the ability of AC VI to traffic to the plasma membrane. Furthermore, mutant expression resulted in a significant reduction in adenylyl cyclase activity. The decrease in AC VI membrane expression was not caused by alterations in enzyme transcription. The effect of the mutant was specific for the AC V and VI isoforms and expression of the transmembrane M1 domain but not the C1a domain was required for the mutant to affect adenylyl cyclase activity. In aggregate, these data suggest that alterations in the ability of adenylyl cyclases to form higher order forms regulate both enzyme trafficking and enzyme activity.

3.597 Fractionation of the epithelial apical junctional complex: reassessment of protein distributions in different substructures

Vogelmann, R. And Nelson, W.J.
*Mol. Biol. Cell, **16**, 701-716 (2005)*

The epithelial apical junctional complex (AJC) is an important regulator of cell structure and function. The AJC is compartmentalized into substructures comprising the tight and adherens junctions, and other membrane complexes containing the membrane proteins nectin, junctional adhesion molecule, and crumbs. In addition, many peripheral membrane proteins localize to the AJC. Studies of isolated proteins indicate a complex map of potential binding partners in which there is extensive overlap in the interactions between proteins in different AJC substructures. As an alternative to a direct search for specific protein-protein

interactions, we sought to separate membrane substructures of the AJC in **iodixanol** density gradients and define their protein constituents. Results show that the AJC can be fractured into membrane substructures that contain specific membrane and peripheral membrane proteins. The composition of each substructure reveals a more limited overlap in common proteins than predicted from the inventory of potential interactions; some of the overlapping proteins may be involved in stepwise recruitment and assembly of AJC substructures.

3.598 Roles of the ITAM and PY motifs of Epstein-Barr virus latent membrane protein 2A in the inhibition of epithelial cell differentiation and activation of β-catenin signaling
Morrison, J.A. and Raab-Traub, N.
J. Virol., ***79(4)***, *2375-2382 (2005)*

Epstein-Barr virus (EBV) latent membrane protein 2A (LMP2A) is important for maintenance of latency in infected B lymphocytes. Through its immunoreceptor tyrosine-based activation motif (ITAM) and PY motifs, LMP2A is able to block B-cell receptor (BCR) signaling, bind BCR-associated kinases, and manipulate the turnover of itself and these kinases via a PY-mediated interaction with the Nedd4 family of ubiquitin ligases. In epithelial cells, LMP2A has been shown to activate the phosphatidylinositol 3'-OH kinase/Akt and ß-catenin signaling pathways. In the present study, the biological consequences of LMP2A expression in the normal human foreskin keratinocyte (HFK) cell line were investigated and the importance of the ITAM and PY motifs for LMP2A signaling effects in HFK cells was ascertained. The ITAM was essential for the activation of Akt by LMP2A in HFK cells, while both the ITAM and PY motifs contributed to LMP2A-mediated accumulation and nuclear translocation of the oncoprotein ß-catenin. LMP2A inhibited induction of differentiation in an assay conducted with semisolid methylcellulose medium, and the PY motifs were critical for this inhibition. LMP2A is expressed in the EBV-associated epithelial malignancies nasopharyngeal carcinoma and gastric carcinoma, and these data indicate that LMP2A affects cellular processes that likely contribute to carcinogenesis.

3.599 Alteration of the extracellular matrix interferes with raft association of neurofascin in oligodendrocytes. Potential significance for multiple sclerosis
Maier, O. Et al
Mol. Cell. Neurosci., ***28(2)***, *390-401 (2005)*

Remyelination, as potential treatment for demyelinating diseases like multiple sclerosis (MS), requires the formation of new axoglial interactions by differentiating oligodendrocyte progenitor cells. Since the oligodendrocyte-specific isoform of neurofascin, NF155 (neurofascin isoform of 155 kDa), may be important for establishing axoglial interactions, we analyzed whether its expression is changed in chronic relapsing experimental allergic encephalomyelinitis (EAE). Although overall expression of NF155 was not changed, immunoreactivity of NF155 was dramatically increased in EAE lesion sites indicating an enhanced accessibility of NF155 epitopes. As this may be due to infiltrating plasma components, for example, fibronectin, we analyzed whether fibronectin affects the intracellular distribution and membrane association of NF155 in primary oligodendrocytes. In oligodendrocytes cultivated on polylysine, NF155 was recruited to membrane microdomains (rafts) during development and became enriched in secondary and tertiary processes. Fibronectin perturbed localization and raft association of NF155 and inhibited the morphological differentiation of oligodendrocytes. Consistent with the in vitro data, raft association of NF155 was reduced in spinal cord of EAE rats. The results suggest that the association of NF155 to microdomains in the oligodendrocyte membrane is required for its participation in intermolecular interactions, which are important for myelination and/or myelin integrity.

3.600 Characterizing the sphingolipid signaling pathway that remediates defects associated with loss of the yeast amphiphysin-like orthologs, Rvs161p and Rvs167p
Germann, M., Swain, E., Bergman, L. and Nickels, Jr. J.T.
J. Biol. Chem., ***280(6)***, *4270-4278 (2005)*

Loss of function of either the *RVS161* or *RVS167 Saccharomyces cerevisiae* amphiphysin-like gene confers similar growth phenotypes that can be suppressed by mutations in sphingolipid biosynthesis. We performed a yeast two-hybrid screen using Rvs161p as bait to uncover proteins involved in this sphingolipid-dependent suppressor pathway. In the process, we have demonstrated a direct physical interaction between Rvs167p and the two-hybrid interacting proteins, Acf2p, Gdh3p, and Ybr108wp, while also elucidating the Rvs167p amino acid domains to which these proteins bind. By using subcellular fractionation, we demonstrate that Rvs167p, Ybr108wp, Gdh3p, and Acf2p all localize to Rvs161p-containing lipid rafts,

thus placing them within a single compartment that should facilitate their interactions. Moreover, our results suggest that Acf2p and Gdh3p functions are needed for suppressor pathway activity. To determine pathway mechanisms further, we examined the localization of Rvs167p in suppressor mutants. These studies reveal roles for Rvs161p and the very long chain fatty acid elongase, Sur4p, in the localization and/or stability of Rvs167p. Previous yeast studies showed that *rvs* defects could be suppressed by changes in sphingolipid metabolism brought about by deleting *SUR4* (Desfarges, L., Durrens, P., Juguelin, H., Cassagne, C., Bonneu, M., and Aigle, M. (1993) *Yeast* 9, 267–277). Using *rvs167 sur4* and *rvs161 sur4* double null cells as models to study suppressor pathway activity, we demonstrate that loss of *SUR4* does not remediate the steady-state actin cytoskeletal defects of *rvs167* or *rvs161* cells. Moreover, suppressor activity does not require the function of the actin-binding protein, Abp1p, or Sla1p, a protein that is thought to regulate assembly of the cortical actin cytoskeleton. Based on our results, we suggest that sphingolipid-dependent suppression of *rvs* defects may not work entirely through regulating changes in actin organization.

3.601 Internalization of renal type Iic Na-P_i cotransporter in response to a high-phosphate diet
Segawa, H. et al
*Am. J. Renal Physiol., **288**, F587-F596 (2005)*

Dietary phosphate levels regulate the renal brush-border type IIa Na-P_i cotransporter. Another Na-P_i cotransporter, type IIc, colocalizes with type IIa Na-P_i cotransporter in the apical membrane of renal proximal tubular cells. The goal of the present study was to determine whether dietary phosphate levels also rapidly regulate the type IIc Na-P_i cotransporter. Type IIa and type IIc transporter protein levels were increased in rats chronically fed a low-P_i diet compared with those fed a normal-P_i diet. Two hours after beginning a high-P_i diet, type IIa transporter levels were decreased, whereas type IIc protein levels remained unchanged. Western blot analysis of brush-border membrane prepared 4 h after beginning a high-P_i diet showed a significant reduction in type IIc transporter protein levels, and immunohistochemistry showed translocation of the type IIc-immunoreactive signal from the entire brush border to subapical membrane. Membrane fractionation studies revealed a decrease in apical membrane type IIc protein without changes in total cortical type IIc protein, which is compatible with redistribution of type IIc protein from the apical membrane to the dense membrane fraction. The microtubule-disrupting reagent colchicine prevented this reduction in apical type IIc transporter at the apical membrane but had no effect on type IIa transporter levels. These data suggest that the type IIc Na-P_i cotransporter level is rapidly regulated by rapid adaptation to dietary P_i in a microtubule-dependent manner. Furthermore, the mechanisms of the internalization of the type IIc transporter are distinct from those of the type IIa transporter.

3.602 Phosphorylation-induced autoinhibition regulates the cytoskeletal protein lethal (2) giant larvae
Betschinger, J., Eisenhaber, F. and Knoblich, J.A.
*Current Biol., **15(3)**, 276-282 (2005)*

During asymmetric cell division, cell fate determinants localize asymmetrically and segregate into one of the two daughter cells. In *Drosophila* neuroblasts, the asymmetric localization of cell fate determinants to the basal cell cortex requires aPKC. aPKC localizes to the apical cell cortex and phosphorylates the cytoskeletal protein Lethal (2) giant larvae (Lgl). Upon phosphorylation, Lgl dissociates from the cytoskeleton and becomes inactive. Here, we show that phosphorylation regulates Lgl by allowing an autoinhibitory interaction of the N terminus with the C terminus of the protein. We demonstrate that interaction with the cytoskeleton is mediated by a C-terminal domain while the N terminus is not required. Instead, the N terminus can bind to the C terminus and can compete for binding to the cytoskeleton. Interaction between the N- and C-terminal domains requires phosphorylation of Lgl by aPKC. Our results suggest that unphosphorylated, active Lgl exists in an open conformation that interacts with the cytoskeleton while phosphorylation changes the protein to an autoinhibited state.

3.603 Annexin 2 promotes the formation of lipid microdomains required for calcium-regulated exocytosis of dense-core vesicles
Chasserot-Golaz, S. et al
*Mol. Biol. Cell, **16**, 1108-1119 (2005)*

Annexin 2 is a calcium-dependent phospholipid-binding protein that has been implicated in a number of membranerelated events, including regulated exocytosis. In chromaffin cells, we previously reported that catecholamine secretion requires the translocation and formation of the annexin 2 tetramer near the exocytotic sites. Here, to obtain direct evidence for a role of annexin 2 in exocytosis, we modified its

expression level in chromaffin cells by using the Semliki Forest virus expression system. Using a real-time assay for individual cells, we found that the reduction of cytosolic annexin 2, and the consequent decrease of annexin 2 tetramer at the cell periphery, strongly inhibited exocytosis, most likely at an early stage before membrane fusion. Secretion also was severely impaired in cells expressing a chimera that sequestered annexin 2 into cytosolic aggregates. Moreover, we demonstrate that secretagogue-evoked stimulation triggers the formation of lipid rafts in the plasma membrane, essential for exocytosis, and which can be attributed to the annexin 2 tetramer. We propose that annexin 2 acts as a calcium-dependent promoter of lipid microdomains required for structural and spatial organization of the exocytotic machinery.

3.604 The deubiquitinating enzyme Ubp1 affects sorting of the ATP-binding cassette-transporter Ste6 in the endocytic pathway
Schmitz, C., Kinner, A. and Kölling, R.
*Mol. Biol. Cell, **16**, 1319-1329 (2005)*

Deubiquitinating enzymes (Dubs) are potential regulators of ubiquitination-dependent processes. Here, we focus on a member of the yeast ubiquitin-specific processing protease (Ubp) family, the Ubp1 protein. We could show that Ubp1 exists in two forms: a longer membrane-anchored form (mUbp1) and a shorter soluble form (sUbp1) that seem to be independently expressed from the same gene. The membrane-associated mUbp1 variant could be localized to the endoplasmic reticulum (ER) membrane by sucrose density gradient centrifugation and by immunofluorescence microscopy. Overexpression of the soluble Ubp1 variant stabilizes the ATP-binding cassette-transporter Ste6, which is transported to the lysosome-like vacuole for degradation, and whose transport is regulated by ubiquitination. Ste6 stabilization was not the result of a general increase in deubiquitination activity, because overexpression of Ubp1 had no effect on the degradation of the ER-associated degradation substrate carboxypeptidase Y^* and most importantly on Ste6 ubiquitination itself. Also, overexpression of another yeast Dub, Ubp3, had no effect on Ste6 turnover. This suggests that the Ubp1 target is a component of the protein transport machinery. On Ubp1 overexpression, Ste6 accumulates at the cell surface, which is consistent with a role of Ubp1 at the internalization step of endocytosis or with enhanced recycling to the cell surface from an internal compartment.

3.605 A domain at the C-terminus of PS1 is required for presenilinase and γ-secretase activities
Brunkan, A.L., Martinez, M., Wang, J., Walker, E.S. and Goate, A.M.
*J. Neurochem., **92(5)**, 1158-1169 (2005)*

The structural requirements for presenilin (PS) to produce active presenilinase and γ-secretase enzymes are poorly understood. Here we investigate the role the cytoplasmic C-terminal region of PS1 plays in PS1 activity. Deletion or addition of residues at the PS C-terminus has been reported to inhibit presenilinase endoproteolysis of PS and alter γ-secretase activity. In this study, we use a sensitive assay in PS1/2KO MEFs to define a domain at the extreme C-terminus of PS1 that is essential for both presenilinase and γ-secretase activities. Progressive deletion of the C-terminus demonstrated that removal of nine residues produces a PS1 molecule (458ST) that lacks both presenilinase processing and γ-secretase cleavage of Notch and APP substrates. In contrast, removal of four or five residues had no effect (462ST, 463ST), while intermediate truncations partially inhibited PS1 activity. The 458ST mutant was unable to replace endogenous wtPS1 in HEK293 cells. Although 458ST was able to form a γ-secretase complex, this complex was not matured, illustrated by mutant PS1 instability, lack of endoproteolysis, and little production of mature Nicastrin. These data indicate that the C-terminal end of PS1 is essential for Nicastrin trafficking and modification as well as the replacement of endogenous PS1 by PS1 transgenes.

3.606 Novel role of ARF6 in vascular endothelial growth factor-induced signaling and angiogenesis
Ikeda, S. et al
*Cir. Res., **96**, 467-475 (2005)*

Vascular endothelial growth factor (VEGF) stimulates endothelial cell (EC) migration and proliferation primarily through the VEGF receptor-2 (VEGFR2). We have shown that VEGF stimulates a Rac1-dependent NAD(P)H oxidase to produce reactive oxygen species (ROS) that are involved in VEGFR2 autophosphorylation and angiogenic-related responses in ECs. The small GTPase ARF6 is involved in membrane trafficking and cell motility; however, its roles in VEGF signaling and physiological responses in ECs are unknown. In this study, we show that overexpression of dominant-negative ARF6 [ARF6(T27N)] almost completely inhibits VEGF-induced Rac1 activation, ROS production, and VEGFR2 autophosphorylation in ECs. Fractionation of caveolae/lipid raft membranes demonstrates that ARF6, Rac1,

and VEGFR2 are localized in caveolin-enriched fractions basally. VEGF stimulation results in the release of VEGFR2 from caveolae/lipid rafts and caveolin-1 without affecting localization of ARF6, Rac1, or caveolin-1 in these fractions. The egress of VEGFR2 from caveolae/lipid rafts is contemporaneous with the tyrosine phosphorylation of caveolin-1 (Tyr14) and VEGFR2 and with their association with each other. ARF6(T27N) significantly inhibits both VEGF-induced responses. Immunofluorescence studies show that activated VEGFR2 and phosphocaveolin colocalize at focal complexes/adhesions after VEGF stimulation. Both overexpression of ARF6(T27N) and mutant caveolin-1(Y14F), which cannot be phosphorylated, block VEGF-stimulated EC migration and proliferation. Moreover, ARF6 expression is markedly upregulated in association with an increase in capillary density in a mouse hindlimb ischemia model of angiogenesis. Thus, ARF6 is involved in the temporal-spatial organization of caveolae/lipid rafts– and ROS-dependent VEGF signaling in ECs as well as in angiogenesis in vivo.

3.607 Stable plasma membrane levels of hCTR1 mediate cellular copper uptake
Eisses, J.F., Chi, Y. and Kaplan, J.H.
J. Biol. Chem., ***280(10)***, *9635-9639 (2005)*

The human copper transporter 1 (hCtr1), when heterologously overexpressed in insect cells, mediates saturable Cu uptake. In mammalian expression systems, a rapid Cu-dependent internalization of hCtr1 has been reported in cells that overexpress epitope-tagged hCtr1 when exposed to Cu in the external medium. This finding led to the suggestion that such internalization may be a step in the hCtr1 transmembrane Cu transport mechanism. We have demonstrated that preincubation in Cu-containing media of *sf9* cells stably expressing hCtr1 has no effect on the initial rate of Cu transport. Furthermore, Western blot analyses of fractionated *sf9* cell membranes show no evidence of a regulatory Cu-dependent internalization from the plasma membrane. In similar studies on human embryonic kidney (HEK) 293 cells, we showed that incubation with Cu does not alter the initial rate of Cu uptake mediated by endogenous levels of hCtr1 compared with untreated cells. Confirmation that hCtr1 mediates this transport is provided by specific small interfering RNA-dependent decreases in hCtr1 protein levels and in Cu transport rates. Western blot analysis and confocal microscopy of human embryonic kidney 293 cells showed that the majority of hCtr1 protein is localized at the plasma membrane and no significant internalization is detected upon Cu treatment. We concluded that internalization of hCtr1 is not a required step in the transport pathway; we suggest that oligomeric hCtr1 acts as a conventional transporter providing a permeation pathway for Cu through the membrane and that internalization of endogenous hCtr1 in response to elevated extracellular Cu levels does not play a significant regulatory role in Cu homeostasis.

3.608 Membrane localization of the U2 domain of protein 4.1B is necessary and sufficient for meningioma growth suppression
Robb, V.A., Gerber, M.A., Hart-Mahon, E.K. and Gutmann, D.H.
Oncogene, ***24****, 1946-1957 (2005)*

Meningiomas are common central nervous system tumors; however, the molecular mechanisms underlying their pathogenesis are largely undefined. Previous work has implicated Protein 4.1B as an important tumor suppressor involved in the development of these neoplasms. In this report, we demonstrate that the U2 domain is necessary and sufficient for the ability of Protein 4.1B to function as a meningioma growth suppressor. Using a series of truncation and deletion constructs of DAL-1 (a fragment of Protein 4.1B that retains all the growth suppressive properties), we narrowed the domain required for 4.1B growth suppression to a fragment containing a portion of the FERM domain and the U2 domain using clonogenic assays on meningioma cells. Deletion of the U2 domain in the context of the full-length DAL-1 molecule eliminated growth suppressor function, as measured by thymidine incorporation and caspase-3 activation. Moreover, targeting the U2 domain to the plasma membrane using a membrane localization signal (MLS) reduced cell proliferation, similar to wild-type DAL-1. Collectively, the data suggest that the U2 domain, when properly targeted to the plasma membrane, contains all the residues necessary for mediating Protein 4.1B growth suppression.

3.609 Nuclear location-dependent role of peripheral benzodiapine receptor (PBR) in hepatic tumoral cell lines proliferation
Corsi, L., Geminiani, E., Avallone, R. and Baraldi, M.
Life Sciences, ***76(22)****, 2523-2533 (2005)*

PBR is involved in numerous biological functions, including steroid biosynthesis, mitochondrial oxidative phosphorylation and cell proliferation. The presence of PBR at the perinuclear/nuclear subcellular level

has been demonstrated in aggressive breast cancer cell lines and human glioma cells where it seems to be involved in cell proliferation. In our study we investigated the presence of perinuclear/nuclear PBR in different hepatic tumor cell lines with regard to binding to [^{3}H] PK 11195 and protein analysis. The results obtained by saturation binding experiments and scatchard analysis of perinuclear/nuclear PBR density in parallel with the results on the growth curves of the cell lines tested, indicate that the perinuclear/nuclear PBR density correlates inversely with cell doubling time. Moreover, the cell line with high perinuclear/nuclear PBR proliferated in response to PBR ligand, whereas that with low perinuclear/nuclear PBR did not. Our results reinforce the idea that the subcellular localisation of PBR defines its function and that this receptor could be a possible target for new strategies against cancer.

3.610 Two modes of exocytosis at hippocampal synapses revealed by rate of FM1-43 efflux from individual vesicles
Richards, D.A., Bai, J. and Chapman, E.R.
J. Cell Biol., ***168(6)***, *929-939 (2005)*

We have examined the kinetics by which FM1-43 escapes from individual synaptic vesicles during exocytosis at hippocampal boutons. Two populations of exocytic events were observed; small amplitude events that lose dye slowly, which made up more than half of all events, and faster, larger amplitude events with a fluorescence intensity equivalent to single stained synaptic vesicles. These populations of destaining events are distinct in both brightness and kinetics, suggesting that they result from two distinct modes of exocytosis. Small amplitude events show tightly clustered rate constants of dye release, whereas larger events have a more scattered distribution. Kinetic analysis of the association and dissociation of FM1-43 with membranes, in combination with a simple pore permeation model, indicates that the small, slowly destaining events may be mediated by a narrow ~1-nm fusion pore.

3.611 Neutral sphingomyelinase 2 (*smpd3*) in the control of postnatal growth and development
Stoffel, W., Jenke, B., Blöck, B., Zumbansen, M. And Koebke, J.
PNAS, ***102(12)***, *4554-4559 (2005)*

Neutral sphingomyelinases sphingomyelin phosphodiesterase (SMPD)2 and -3 hydrolyze sphingomyelin to phosphocholine and ceramide. *smpd2* is expressed ubiquitously, and *smpd3* is expressed predominantly in neurons of the CNS. Their activation and the functions of the released ceramides have been associated with signaling pathways in cell growth, differentiation, and apoptosis. However, these cellular responses remain poorly understood. Here we describe the generation and characterization of the *smpd3*$^{-/-}$ and *smpd2*$^{-/-}$ *smpd3*$^{-/-}$ double mutant mouse, which proved to be devoid of neutral sphingomyelinase activity. SMPD3 plays a pivotal role in the control of late embryonic and postnatal development: the *smpd3*-null mouse develops a novel form of dwarfism and delayed puberty as part of a hypothalamus-induced combined pituitary hormone deficiency. Our studies suggest that SMPD3 is segregated into detergent-resistant subdomains of Golgi membranes of hypothalamic neurosecretory neurons, where its transient activation modifies the lipid bilayer, an essential step in the Golgi secretory pathway. The *smpd3*$^{-/-}$ mouse might mimic a form of human combined pituitary hormone deficiency.

3.612 Caveolae targeting and regulation of large conductance Ca^{2+} -activated K^+ channels in vascular endothelial cells
Wang, X-L. et al
J. Biol. Chem., ***280(12)***, *11656-11664 (2005)*

The vascular endothelium is richly endowed with caveolae, which are specialized membrane microdomains that facilitate the integration of specific cellular signal transduction processes. We found that the large conductance Ca^{2+}-activated K^+ (BK) channels are associated with caveolin-1 in bovine aortic endothelial cells (BAECs). **OptiPrep** gradient cell fractionation demonstrated that BK channels were concentrated in the caveolae-rich fraction in BAECs. Immunofluorescence imaging showed co-localization of caveolin-1 and BK channels in the BAEC membrane. Immunoprecipitation and glutathione *S*-transferase pull-down assay results indicated that caveolin-1 and BK channels are physically associated. However, whole cell patch clamp recordings could not detect BK (iberiotoxin-sensitive) currents in cultured BAECs under baseline conditions, even though the presence of BK mRNA and protein expression was confirmed by reverse transcription-PCR and Western blots. Cholesterol depletion redistributed the BK channels to non-caveolar fractions of BAECs, resulting in BK channel activation (7.3 ± 1.6 pA/picofarad (pF), n = 5). BK currents were also activated by isoproterenol (ISO, 1 μM, 6.9 ± 2.4 pA/pF, n = 6). Inclusion of a caveolin-1 scaffolding domain peptide (10 μM) in the pipette solution completely abrogated

the effects of ISO on BK channel activation, whereas inclusion of the scrambled control peptide (10 μM) did not inhibit the ISO effects. We have also found that caveolin-1 knockdown by small interference RNA activated BK currents (5.3 ± 1.4 pA/pF, $n = 6$). We conclude that: 1) BK channels are targeted to caveolae microdomains in vascular endothelial cells; 2) caveolin-1 interacts with BK channels and exerts a negative regulatory effect on channel functions; and 3) BK channels are inactive under control conditions but can be activated by cholesterol depletion, knockdown of caveolin-1 expression, or ISO stimulation. These novel findings may have important implications for the role of BK channels in the regulation of endothelial function.

3.613 Aph-1 contributes to the stabilization and trafficking of the γ-secretase complex through mechanisms involving intermolecular and intramolecular interactions
Niimura, M. Et al
J. Biol. Chem., ***280(13)***, *12967-12975 (2005)*

γ-Secretase cleaves type I transmembrane proteins, including β-amyloid precursor protein and Notch, and requires the formation of a protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2 for its activity. Aph-1 is implicated in the stabilization of this complex, although its precise mechanistic role remains unknown. Substitution of the first glycine within the transmembrane G*XXX*G motif of Aph-1 causes a loss-of-function phenotype in *Caenorhabditis elegans*. Here, using an untranslated region-targeted RNA interference/rescue strategy in *Drosophila* Schneider 2 cells, we show that Aph-1 contributes to the assembly of the γ-secretase complex by multiple mechanisms involving intermolecular and intramolecular interactions depending on or independent of the conserved glycines. Aph-1 binds to nicastrin forming an early subcomplex independent of the conserved glycines within the endoplasmic reticulum. Certain mutations in the conserved G*XXX*G motif affect the interaction of the Aph-1·nicastrin subcomplex with presenilin that mediates trafficking of the presenilin·Aph-1·nicastrin tripartite complex to the Golgi. The same mutations decrease the stability of Aph-1 polypeptides themselves, possibly by affecting intramolecular associations through the transmembrane domains. Our data suggest that the proper assembly of the Aph-1·nicastrin subcomplex with presenilin is the prerequisite for the trafficking as well as the enzymatic activity of the γ-secretase complex and that Aph-1 functions as a stabilizing scaffold in the assembly of this complex.

3.614 Molecular mechanisms of cholesterol absorption and transport in the intestine
Hui, D.Y. and Howles, P.N.
Seminars in Cell & Developmental Biol., ***16(2)***, *183-192 (2005)*

Many enzymes and transport proteins participate in cholesterol absorption. This review summarizes recent results on several proteins that are important for each step of the cholesterol absorption pathway, including the important roles of: (i) pancreatic triglyceride lipase (PTL), carboxyl ester lipase (CEL), and ileal bile acid transporter in determining the rate of cholesterol absorption; (ii) ATP binding cassette (ABC) transporters and the Niemann-Pick C-1 like-1 (NPC1L1) protein as intestinal membrane gatekeepers for cholesterol efflux and influx; and (iii) intracellular membrane vesicles and transport proteins in lipid trafficking through intracellular compartments prior to lipoprotein assembly and secretion to plasma circulation.

3.615 Adeno-associated virus-mediated gene transfer of the heart/muscle adenine nucleotide translocator (ANT) in mouse
Flierl, A., Chen, Y., Coskun, P.E., Samulski, R.J. and Wallace, D.C.
Gen. Ther., ***12***, *570-578 (2005)*

Mitochondrial myopathy, associated with muscle weakness and progressive external ophthalmoplegia, is caused by mutations in mitochondria oxidative phosphorylation genes including the heart–muscle isoform of the mitochondrial adenine nucleotide translocator (ANT1). To develop therapies for mitochondrial disease, we have prepared a recombinant adeno-associated viral vector (rAAV) carrying the mouse Ant1 cDNA. This vector has been used to transduce muscle cells and muscle from Ant1 mutant mice, which manifest mitochondrial myopathy. AAV-ANT1 transduction resulted in long-term, stable expression of the Ant1 transgene in muscle precursor cells as well as differentiated muscle fibers. The transgene ANT1 protein was targeted to the mitochondrion, was inserted into the mitochondrial inner membrane, formed a functional ADP/ATP carrier, increased the mitochondrial export of ATP and reversed the histopathological changes associated with the mitochondrial myopathy. Thus, AAV transduction has the potential of

providing symptomatic relief for the ophthalmoplegia and ptosis resulting from paralysis of the extraocular eye muscles cause by mutations in the Ant1 gene.

3.616 Nulear localization anti-DNA antibodies enter cells via caveoli and modulate expression of caveolin and p53

Yanase, K. and Madaio, M.P.
J. Autoimmun., ***24****, 145-151 (2005)*

After administration to normal mice, a subset of monoclonal (m) anti-DNA antibodies (Ab) derived from MRL-*lpr/lpr* mice was identified that enter cells, in vivo. In the kidneys, this was associated with glomerular hypercellularity and proteinuria. In cultured cells, the same mAb bound to myosin 1 on the cell surface, prior to internalization, nuclear localization and inhibition of apoptosis. The present study focuses on the mechanisms underlying the observed functional effects. Subcellular localization studies revealed that following internalization, a prototypic, nuclear localizing, m antibody (Ab; termed H7) co-localized with myosin 1, shortly after internalization, within caveolae, near the cell membrane. Cell fractionation studies confirmed the presence of both H7 and myosin within the caveolar fraction. Since variations in caveolin protein expression have been associated with apoptotic events in cancer cells, through p53 dependent and independent pathways, modulation of caveolin by intracellular H7 was evaluated. Cellular entry of the anti-DNA Ab resulted in an increase in caveolin protein expression. Furthermore, after exposure of cells to dexamethasone to induce apoptosis, the usual increase in p53 was inhibited in the presence of intracellular H7. Taken together, the results suggest that upregulation of caveolin and inhibition of p53 induction are involved in H7-induced, inhibition of apoptosis. Furthermore, they suggest that this inhibition contributes to the glomerular hypercellularity observed in normal mice with intranuclear H7. The results also raise the possibility that inhibition of apoptotic pathways during inflammation or/and autoimmunity could influence subsequent disease events. The novel mechanism of cellular perturbation is indirect and dependent on apoptotic stimuli, and it may account for the presence of intranuclear antibodies in inflammatory and normal tissues of individuals with lupus.

3.617 A simplified method for the preparation of detergent-free lipid rafts

Macdonald, J.L. and Pike, L.J.
J. Lipid. Res., ***46****, 1061-1067 (2005)*

Lipid rafts are small plasma membrane domains that contain high levels of cholesterol and sphingolipids. Traditional methods for the biochemical isolation of lipid rafts involve the extraction of cells with nonionic detergents followed by the separation of a low-density, detergent-resistant membrane fraction on density gradients. Because of concerns regarding the possible introduction of artifacts through the use of detergents, it is important to develop procedures for the isolation of lipid rafts that do not involve detergent extraction. We report here a simplified method for the purification of detergent-free lipid rafts that requires only one short density gradient centrifugation, but yields a membrane fraction that is highly enriched in cholesterol and protein markers of lipid rafts, with no contamination from nonraft plasma membrane or intracellular membranes.

3.618 Studies on rabbit natural and recombinant tissue factors: intracellular retention and regulation of surface expression in cultured cells

Fortin, J-P., Rivard, G.E., Adam, A. and Marceau, F.
Am. J. Physiol., ***288****, H2192-H2202 (2005)*

Tissue factor (TF) is the most important trigger of blood coagulation in vascular pathology. Rabbit TF, with or without (ΔC) its COOH-terminal intracellular tail, has been conjugated to green fluorescent protein (GFP) to study subcellular localization and other functions of TF. TF-GFP and TFΔC-GFP are associated with Na_2CO_3-resistant buoyant fractions in HEK-293 cells (lipid rafts); there is no morphological difference in the surface distribution of these or other GFP-labeled membrane proteins present in or excluded from rafts (confocal microscopy, HEK-293 cells). Endogenous TF expressed by rabbit aortic smooth muscle cells (SMCs) is also raft associated. Membranes from HEK-293 cells expressing recombinant TF-GFP or wild-type TF were equipotent to clot human plasma; however, TFΔC-GFP was ~ 20-fold more active (per membrane weight). Immunoblot confirmed that the deletion mutant is more abundantly expressed, and confocal microscopy showed that it has preferential membrane localization, whereas TF-GFP is mainly intracellular (nuclear lining and multiple granules). With a similar half-life (<4 h), the two constructions differ by their intracellular retention, lower for TFΔC-GFP. In serum-starved SMCs, the expression of endogenous TF was upregulated by interleukin-1β and/or FBS treatment

(immunoblot, immunofluorescence, clotting assay). However, TF secretion or surface expression was not regulated by stimuli of physiological intensity (such as stimulation of the coexpressed kinin B_1 receptors), although a calcium ionophore was highly active in this respect. TF is a raft-associated molecule whose surface expression (secretion) is apparently retarded or impaired by structural determinant(s) located in its COOH-terminal tail.

3.619 Proteomic profiling of hepatic endoplasmic reticulum-associated proteins in an animal model of insulin resistance and metabolic dyslipidemia
Morand, J-P. F., Macri, J. and Adeli, K.
J. Biol. Chem., ***280(18)***, *17626-17633 (2005)*

Hepatic insulin resistance and lipoprotein overproduction are common features of the metabolic syndrome and insulin-resistant states. A fructose-fed, insulin-resistant hamster model was recently developed to investigate mechanisms linking the development of hepatic insulin resistance and overproduction of atherogenic lipoproteins. Here we report a systematic analysis of protein expression profiles in the endoplasmic reticulum (ER) fractions isolated from livers of fructose-fed hamsters with the intention of identifying new candidate proteins involved in hepatic complications of insulin resistance and lipoprotein dysregulation. We have profiled hepatic ER-associated proteins from chow-fed (control) and fructose-fed (insulin-resistant) hamsters using two-dimensional gel electrophoresis and mass spectrometry. A total of 26 large scale two-dimensional gels of hepatic ER were used to identify 34 differentially expressed hepatic ER protein spots observed to be at least 2-fold differentially expressed with fructose feeding and the onset of insulin resistance. Differentially expressed proteins were identified by matrix-assisted laser desorption ionization-quadrupole time of flight (MALDI-Q-TOF), MALDI-TOF-postsource decay, and database mining using ProteinProspector MS-fit and MS-tag or the PROWL ProFound search engine using a focused rodent or mammalian search. Hepatic ER proteins ER60, ERp46, ERp29, glutamate dehydrogenase, and TAP1 were shown to be more than 2-fold down-regulated, whereas α-glucosidase, P-glycoprotein, fibrinogen, protein disulfide isomerase, GRP94, and apolipoprotein E were all found to be up-regulated in the hepatic ER of the fructose-fed hamster. Seven isoforms of ER60 in the hepatic ER were all shown to be down-regulated at least 2-fold in hepatocytes from fructosefed/insulin-resistant hamsters. Implications of the differential expression of positively identified protein factors in the development of hepatic insulin resistance and lipoprotein abnormalities are discussed.

3.620 The low density lipoprotein receptor-related protein (LRP) is a novel β-secretase (BACE1) substrate
Von Armin, C.A.F. et al
J. Biol. Chem., ***280(18)***, *17777-17785 (2005)*

BACE is a transmembrane protease with β-secretase activity that cleaves the amyloid precursor protein (APP). After BACE cleavage, APP becomes a substrate for γ-secretase, leading to release of amyloid-β peptide (Aβ), which accumulates in senile plaques in Alzheimer disease. APP and BACE are co-internalized from the cell surface to early endosomes. APP is also known to interact at the cell surface and be internalized by the low density lipoprotein receptor-related protein (LRP), a multifunctional endocytic and signaling receptor. Using a new fluorescence resonance energy transfer (FRET)-based assay of protein proximity, fluorescence lifetime imaging (FLIM), and co-immunoprecipitation we demonstrate that the light chain of LRP interacts with BACE on the cell surface in association with lipid rafts. Surprisingly, the BACE-LRP interaction leads to an increase in LRP C-terminal fragment, release of secreted LRP in the media and subsequent release of the LRP intracellular domain from the membrane. Taken together, these data suggest that there is a close interaction between BACE and LRP on the cell surface, and that LRP is a novel BACE substrate.

3.621 Disturbance of sphingolipid biosynthesis abrogates the signaling of Mss4, phosphatidylinositol-4-phosphate 5-kinase, in yeast
Kobayashi, T., Takematsu, H., Yamaji, T., Hiramoto, S. and Kozutsumi, Y.
J. Biol. Chem., ***289(18)***, *18087-18094 (2005)*

The functional relationships between phosphoinositides and sphingolipids have not been well characterized to date. ISP-1/myriocin is a potent inhibitor of sphingolipid biosynthesis and induces severe growth defects in eukaryotic cells because of the sphingolipid deprivation. We characterized a novel multicopy suppressor gene of ISP-1-mediated cell death in yeast, *MSS4*. *MSS4* encodes a phosphatidylinositol-4-phosphate 5-kinase that synthesizes phosphatidylinositol (4,5)-bisphosphate ($PI4,5P_2$). We demonstrate here that ISP-1

treatment of yeast causes defects both in the activity and subcellular localization of Mss4. The effect of the Mss4 defect on the downstream signaling was examined, because interaction between the Mss4 product, $PI4,5P_2$, and the pleckstrin-homology domain of Rom2 mediates recruitment of Rom2 to the membrane, which is the crucial step for subsequent Rho1/2 activation. Indeed, failure of Rom2 recruitment was observed in ISP-1-treated cells as well as in *csg2*-deleted cells, which have reduced mannosylated inositolphosphorylceramide. These data suggested that proper sphingolipids are required for the signaling pathway involving Mss4.

3.622 Actin depolymerization transduces the strength of B-cell receptor stimulation
Hao, S. and August, A.
*Mol. Biol. Cell, **16**, 2275-2294 (2005)*

Polymerization of the actin cytoskeleton has been found to be essential for B-cell activation. We show here, however, that stimulation of BCR induces a rapid global actin depolymerization in a BCR signal strength-dependent manner, followed by polarized actin repolymerization. Depolymerization of actin enhances and blocking actin depolymerization inhibits BCR signaling, leading to altered BCR and lipid raft clustering, ERK activation, and transcription factor activation. Furthermore actin depolymerization by itself induces altered lipid raft clustering and ERK activation, suggesting that F-actin may play a role in separating lipid rafts and in setting the threshold for cellular activation.

3.623 Myosin-1a is critical for normal brush border structure and composition
Tyska, M. Et al
*Mol. Biol. Cell, **16**, 2443-2457 (2005)*

To develop our understanding of myosin-1a function in vivo, we have created a mouse line null for the *myosin-1a* gene. Myosin-1a knockout mice demonstrate no overt phenotypes at the whole animal level but exhibit significant perturbations and signs of stress at the cellular level. Among these are defects in microvillar membrane morphology, distinct changes in brush-border organization, loss of numerous cytoskeletal and membrane components from the brush border, and redistribution of intermediate filament proteins into the brush border. We also observed significant ectopic recruitment of another short-tailed class I motor, myosin-1c, into the brush border of knockout enterocytes. This latter finding, a clear demonstration of functional redundancy among vertebrate myosins-I, may account for the lack of a whole animal phenotype. Nevertheless, these results indicate that myosin-1a is a critical multifunctional component of the enterocyte, required for maintaining the normal composition and highly ordered structure of the brush border.

3.624 Cell cycle-regulated microtubeli-independent organelle division in *Cyanidioschyzon merolae*
Hishida, K., Yagisawa, F., Kuroiwa, H., Nagata, T. and Kuroiwa, T.
*Mol. Biol. Cell, **16**, 2493-2502 (2005)*

Mitochondrial and chloroplast division controls the number and morphology of organelles, but how cells regulate organelle division remains to be clarified. Here, we show that each step of mitochondrial and chloroplast division is closely associated with the cell cycle in *Cyanidioschyzon merolae*. Electron microscopy revealed direct associations between the spindle pole bodies and mitochondria, suggesting that mitochondrial distribution is physically coupled with mitosis. Interconnected organelles were fractionated under microtubule-stabilizing condition. Immunoblotting analysis revealed that the protein levels required for organelle division increased before microtubule changes upon cell division, indicating that regulation of protein expression for organelle division is distinct from that of cytokinesis. At the mitochondrial division site, dynamin stuck to one of the divided mitochondria and was spatially associated with the tip of a microtubule stretching from the other one. Inhibition of microtubule organization, proteasome activity or DNA synthesis, respectively, induced arrested cells with divided but shrunk mitochondria, with divided and segregated mitochondria, or with incomplete mitochondrial division restrained at the final severance, and repetitive chloroplast division. The results indicated that mitochondrial morphology and segregation but not division depend on microtubules and implied that the division processes of the two organelles are regulated at distinct checkpoints.

3.625 Presenilin function and γ-secretase activity
Brunkan, A.L. and Goate, A.M.
*J. Neurochem., **93**, 769-792 (2005)*

Alzheimer's disease (AD) is the most common form of dementia and is characterized pathologically by the accumulation of β-amyloid (Aβ) plaques and neurofibrillary tangles in the brain. Genetic studies of AD first highlighted the importance of the presenilins (PS). Subsequent functional studies have demonstrated that PS form the catalytic subunit of the γ-secretase complex that produces the Aβ peptide, confirming the central role of PS in AD biology. Here, we review the studies that have characterized PS function in the γ-secretase complex in *Caenorhabditis elegans*, mice and in *in vitro* cell culture systems, including studies of PS structure, PS interactions with substrates and other γ-secretase complex members, and the evidence supporting the hypothesis that PS are aspartyl proteases that are active in intramembranous proteolysis. A thorough knowledge of the mechanism of PS cleavage in the context of the γ-secretase complex will further our understanding of the molecular mechanisms that cause AD, and may allow the development of therapeutics that can alter Aβ production and modify the risk for AD.

3.626 Amphotropic murine leukaemia virus envelope protein is associated with cholesterol-rich microdomains

Beer, C., Pedersen, L. and Wirth, M.
Virology J., ***2(36)***, *1-9 (2005)*

Background
Cholesterol-rich microdomains like lipid rafts were recently identified as regions within the plasma membrane, which play an important role in the assembly and budding of different viruses, e.g., measles virus and human immunodeficiency virus. For these viruses association of newly synthesized viral proteins with lipid rafts has been shown.
Results
Here we provide evidence for the association of the envelope protein (Env) of the 4070A isolate of amphotropic murine leukaemia virus (A-MLV) with lipid rafts. Using density gradient centrifugation and immunocytochemical analyses, we show that Env co-localizes with cholesterol, ganglioside GM1 and caveolin-1 in these specific regions of the plasma membrane.
Conclusions
These results show that a large amount of A-MLV Env is associated with lipid rafts and suggest that cholesterol-rich microdomains are used as portals for the exit of A-MLV.

3.627 Role of photoreceptor-specific retinal dehydrogenase in the retinoid cycle in vivo

Maeda, A. et al
J. Biol. Chem., ***280(19)***, *18822-18832 (2005)*

The retinoid cycle is a recycling system that replenishes the 11-*cis*-retinal chromophore of rhodopsin and cone pigments. Photoreceptor-specific retinol dehydrogenase (prRDH) catalyzes reduction of all-*trans*-retinal to all-*trans*-retinol and is thought to be a key enzyme in the retinoid cycle. We disrupted mouse *prRDH* (human gene symbol *RDH8*) gene expression by targeted recombination and generated a homozygous *prRDH* knock-out (*prRDH–/–*) mouse. Histological analysis and electron microscopy of retinas from 6- to 8-week-old *prRDH–/–* mice revealed no structural differences of the photoreceptors or inner retina. For brief light exposure, absence of prRDH did not affect the rate of 11-*cis*-retinal regeneration or the decay of Meta II, the activated form of rhodopsin. Absence of prRDH, however, caused significant accumulation of all-*trans*-retinal following exposure to bright lights and delayed recovery of rod function as measured by electroretinograms and single cell recordings. Retention of all-*trans*-retinal resulted in slight overproduction of A2E, a condensation product of all-*trans*-retinal and phosphatidylethanolamine. We conclude that prRDH is an enzyme that catalyzes reduction of all-*trans*-retinal in the rod outer segment, most noticeably at higher light intensities and prolonged illumination, but is not an essential enzyme of the retinoid cycle.

3.628 Acidocacisomes – conserved from bacteria to man

Docampo, R., de Souza, W., Miranda, K., Rohloff, P. and Moreno, S.N.J.
Nature Rev. Microbiol., ***3***, *251-261 (2005)*

Recent work has shown that acidocalcisomes, which are electron-dense acidic organelles rich in calcium and polyphosphate, are the only organelles that have been conserved during evolution from prokaryotes to eukaryotes. Acidocalcisomes were first described in trypanosomatids and have been characterized in most detail in these species. Acidocalcisomes have been linked with several functions, including storage of cations and phosphorus, polyphosphate metabolism, calcium homeostasis, maintenance of intracellular pH

homeostasis and osmoregulation. Here, we review acidocalcisome ultrastructure, composition and function in different trypanosomatids and other organisms.

3.629 C18:3-GM1a induces apoptosis in Neuro2 cells: enzymatic remodeling of fatty acyl chains of glycosphingolipids
Nakagawa, T. Et al
J. Lipi. Res., ***46***, *1103-1112 (2005)*

GM1a [Galß1-3GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Cer] is known to support and protect neuronal functions. However, we report that α-linolenic acid-containing GM1a (C18:3-GM1a), which was prepared using the reverse hydrolysis reaction of sphingolipid ceramide *N*-deacylase, induced apoptosis in neuronal cells. Intranucleosomal DNA fragmentation, chromatin condensation, and caspase activation, all typical features of apoptosis, were observed when mouse neuroblastoma Neuro2a cells were cultured with C18:3-GM1a but not GM1a containing stearic acid (C18:0) or oleic acid (C18:1). The phenotype of Neuro2a cells induced by C18:3-GM1a was similar to that evoked by lyso-GM1a. However, lyso-GM1a caused a complete disruption of lipid microdomains of Neuro2a cells and hemolysis of sheep erythrocytes, whereas C18:3-GM1a did neither. C18:3-GM1a, but not lyso-GM1a, was found to be abundant in lipid microdomains after the removal of loosely bound GM1a by BSA. The activation of stress-activated protein kinase/c-Jun N-terminal kinase in Neuro2a cells was observed with lyso-GM1a but not C18:3-GM1a. These results indicate that the mechanism of apoptosis induced by C18:3-GM1a is distinct from that caused by lyso-GM1a. This study also clearly shows that fatty acid composition of gangliosides significantly affected their pharmacological activities when added to the cell cultures and suggests why naturally occurring gangliosides do not possess polyunsaturated fatty acids as a major constituent.

3.630 Truncated prion protein and doppel are myelinotoxic in the absence of oligodendrocytic PrP^C
Radovanovic, I. Et al
J. Neurosci., ***25(19)***, *4879-4888 (2005)*

The cellular prion protein PrP^C confers susceptibility to transmissible spongiform encephalopathies, yet its normal function is unknown. Although PrP^C-deficient mice develop and live normally, expression of amino proximally truncated PrP^C (ΔPrP) or of its structural homolog Doppel (Dpl) causes cerebellar degeneration that is prevented by coexpression of full-length PrP^C. We now report that mice expressing ΔPrP or Dpl suffer from widespread leukoencephalopathy. Oligodendrocyte-specific expression of full-length PrP^C under control of the myelin basic protein (MBP) promoter repressed leukoencephalopathy and vastly extended survival but did not prevent cerebellar granule cell (CGC) degeneration. Conversely, neuron-specific PrP^C expression under control of the neuron-specific enolase (NSE) promoter antagonized CGC degeneration but not leukoencephalopathy. PrP^C was found in purified myelin and in cultured oligodendrocytes of both wild-type and MBP-PrP transgenic mice but not in NSE-PrP mice. These results identify white-matter damage as an extraneuronal PrP-associated pathology and suggest a previously unrecognized role of PrP^C in myelin maintenance.

3.631 Differential effect of phosphatidylethanolamine depletion on raft proteins. Further evidence for diversity of rafts in *Saccharomyces cerevisiae*
Opekarova, M., Malinska, K., Novakova, L. and Tanner, W.
Biochim. Biophys. Acta, ***1711(1)***, *87-95 (2005)*

A considerable amount of evidence supports the idea that lipid rafts are involved in many cellular processes, including protein sorting and trafficking. We show that, in this process, also a non-raft lipid, phosphatidylethanolamine (PE), has an indispensable function. The depletion of this phospholipid results in an accumulation of a typical raft-resident, the arginine transporter Can1p, in the membranes of Golgi, while the trafficking of another plasma membrane transporter, Pma1p, is interrupted at the level of the ER. Both these transporters associate with a Triton (TX-100) resistant membrane fraction before their intracellular transport is arrested in the respective organelles. The Can1p undelivered to the plasma membrane is fully active when reconstituted to a PE-containing vesicle system in vitro. We further demonstrate that, in addition to the TX-100 resistance at 4 °C, Can1p and Pma1pa exhibit different accessibility to nonyl glucoside (NG), which points to distinct intimate lipid surroundings of these two proteins. Also, at 20 °C, these two proteins are extracted by TX-100 differentially. The features above suggest that Pma1p and Can1p are associated with different compartments. This is independently supported by the observations made by confocal microscopy. In addition we show that PE is involved in the stability of Can1p–raft association.

3.632 Essential function of *Drosophila Sec6* in apical exocytosis of epithelial photoreceptor cells
Beronja, S. et al
*J. Cell Biol., **169(4)**, 635-646 (2005)*

Polarized exocytosis plays a major role in development and cell differentiation but the mechanisms that target exocytosis to specific membrane domains in animal cells are still poorly understood. We characterized *Drosophila* Sec6, a component of the exocyst complex that is believed to tether secretory vesicles to specific plasma membrane sites. *sec6* mutations cause cell lethality and disrupt plasma membrane growth. In developing photoreceptor cells (PRCs), Sec6 but not Sec5 or Sec8 shows accumulation at adherens junctions. In late PRCs, Sec6, Sec5, and Sec8 colocalize at the rhabdomere, the light sensing subdomain of the apical membrane. PRCs with reduced Sec6 function accumulate secretory vesicles and fail to transport proteins to the rhabdomere, but show normal localization of proteins to the apical stalk membrane and the basolateral membrane. Furthermore, we show that Rab11 forms a complex with Sec5 and that Sec5 interacts with Sec6 suggesting that the exocyst is a Rab11 effector that facilitates protein transport to the apical rhabdomere in *Drosophila* PRCs.

3.633 Myosin Va transports dense core secretory vesicles in pancreatic MIN6 β-cells
Varadi, A., Tsuboi, T. And Rutter, G.A.
*Mol. Biol. Cell, **16**, 2670-2680 (2005)*

The role of unconventional myosins in neuroendocrine cells is not fully understood, with involvement suggested in the movement of both secretory vesicles and mitochondria. Here, we demonstrate colocalization of myosin Va (MyoVa) with insulin in pancreatic β-cells and show that MyoVa copurifies with insulin in density gradients and with the vesicle marker phogrin-enhanced green fluorescent protein upon fluorescence-activated sorting of vesicles. By contrast, MyoVa immunoreactivity was poorly colocalized with mitochondrial or other markers. Demonstrating an important role for MyoVa in the recruitment of secretory vesicles to the cell surface, a reduction of MyoVa protein levels achieved by RNA interference caused a significant decrease in glucose- or depolarization-stimulated insulin secretion. Similarly, expression of the dominant-negative–acting globular tail domain of MyoVa decreased by ~50% the number of vesicles docked at the plasma membrane and by 87% the number of depolarization-stimulated exocytotic events detected by total internal reflection fluorescence microscopy. We conclude that MyoVa-driven movements of vesicles along the cortical actin network are essential for the terminal stages of regulated exocytosis in β-cells.

3.634 Acute and chronic changes in cholesterol modulate Na-P_i cotransport activity in OK cells
Breusegem, S.Y. et al
*Am. J. Physiol., **289**, FF154-F165 (2005)*

We previously showed an inverse correlation between membrane cholesterol content and Na-P_i cotransport activity during the aging process and adaptation to alterations in dietary P_i in the rat (Levi M, Jameson DM, and van der Meer BW. *Am J Physiol Renal Fluid Electrolyte Physiol* 256: F85–F94, 1989). The purpose of the present study was to determine whether alterations in cholesterol content per se modulate Na-P_i cotransport activity and apical membrane Na-P_i protein expression in opossum kidney (OK) cells. Acute cholesterol depletion achieved with β-methyl cyclodextrin (β-MCD) resulted in a significant increase in Na-P_i cotransport activity accompanied by a moderate increase in apical membrane Na-P_i protein abundance and no alteration of total cellular Na-P_i protein abundance. Conversely, acute cholesterol enrichment achieved with β-MCD/cholesterol resulted in a significant decrease in Na-P_i cotransport activity with a moderate decrease in apical membrane Na-Pi protein abundance and no change of the total cellular Na-P_i protein abundance. In contrast, chronic cholesterol depletion, achieved by growing cells in lipoprotein-deficient serum (LPDS), resulted in parallel and significant increases in Na-P_i cotransport activity and apical membrane and total cellular Na-P_i protein abundance. Cholesterol depletion also resulted in a significant increase in membrane lipid fluidity and alterations in lipid microdomains as determined by laurdan fluorescence spectroscopy and imaging. Chronic cholesterol enrichment, achieved by growing cells in LPDS followed by loading with low-density lipoprotein, resulted in parallel and significant decreases in Na-P_i cotransport activity and apical membrane and total cellular Na-P_i protein abundance. Our results indicate that in OK cells acute and chronic alterations in cholesterol content per se modulate Na-P_i cotransport activity by diverse mechanisms that also include significant interactions of Na-P_i protein with lipid microdomains.

3.635 Water and solute permeability of rat lung caveolae: high permeabilities explained by acyl chain unsaturation

Hill, W.G., Almasri, E., Ruiz, W.G., Apodaca, G. and Zeidel, M.L.
*Am. J. Physiol., **289**, C33-C41 (2005)*

Caveolae are invaginated membrane structures with high levels of cholesterol, sphingomyelin, and caveolin protein that are predicted to exist as liquid-ordered domains with low water permeability. We isolated a caveolae-enriched membrane fraction without detergents from rat lung and characterized its permeability properties to nonelectrolytes and protons. Membrane permeability to water was $2.85 \pm 0.41 \times 10^{-3}$ cm/s, a value 5–10 times higher than expected based on comparisons with other cholesterol and sphingolipid-enriched membranes. Permeabilities to urea, ammonia, and protons were measured and found to be moderately high for urea and ammonia at $8.85 \pm 2.40 \times 10^{-7}$ and $6.84 \pm 1.03 \times 10^{-2}$ respectively and high for protons at $8.84 \pm 3.06 \times 10^{-2}$ cm/s. To examine whether caveolin or other integral membrane proteins were responsible for high permeabilities, liposomes designed to mimic the lipids of the inner and outer leaflets of the caveolar membrane were made. Osmotic water permeability to both liposome compositions were determined and a combined inner/outer leaflet water permeability was calculated and found to be close to that of native caveolae at $1.58 \pm 1.1 \times 10^{-3}$ cm/s. In caveolae, activation energy for water flux was high (19.4 kcal/mol) and water permeability was not inhibited by $HgCl_2$; however, aquaporin 1 was detectable by immunoblotting. Immunostaining of rat lung with AQP1 and caveolin antisera revealed very low levels of colocalization. We conclude that aquaporin water channels do not contribute significantly to the observed water flux and that caveolae have relatively high water and solute permeabilities due to the high degree of unsaturation in their fatty acyl chains.

3.636 Use of phospho-specific antibodies to determine the phosphorylation of endogenous Na^+/H^+ exchanger NHE3 at PKA consensus sites

Kocinsky, H.S. et al
*Am. J. Physiol., **289**, F249-F258 (2005)*

Transfection studies using mutant constructs have implicated one or both protein kinase A (PKA) consensus phosphorylation sites [serines 552 and 605 in rat Na^+/H^+ exchanger type 3 (NHE3)] as critical for mediating inhibition of NHE3 in response to several stimuli including dopamine. However, whether one or both of these sites is actually phosphorylated in endogenous NHE3 in proximal tubule cells is unknown. The purpose of this study was to generate phosphospecific antibodies so that the state of phosphorylation of these serine residues in endogenous NHE3 could be assessed in vitro and in vivo. To this end, polyclonal and monoclonal phosphospecific peptide antibodies were generated against each PKA consensus site. Phosphospecificity was established by ELISA and Western blot assays. We then used these antibodies in vitro to evaluate the effect of dopamine on phosphorylation of the corresponding PKA sites (serines 560 and 613) in NHE3 endogenously expressed in opossum kidney cells. Baseline phosphorylation of both sites was detected that was significantly increased by dopamine. Next, we determined the baseline phosphorylation state of each serine in rat kidney NHE3 in vivo. We found that serine 552 of NHE3 is phosphorylated to a much greater extent than serine 605 at baseline in vivo. Moreover, we detected a distinct subcellular localization for NHE3 phosphorylated at serine 552 compared with total NHE3. Specifically, NHE3 phosphorylated at serine 552 localized to the coated pit region of the brush-border membrane, where NHE3 is inactive, while total NHE3 was found throughout the brush-border membrane. These findings strongly suggest that phosphorylation of NHE3 plays a role in its subcellular trafficking in vivo. In conclusion, we successfully generated phosphospecific antibodies that should be useful to assess the phosphorylation of endogenous NHE3 at its two PKA consensus sites under a variety of physiological conditions in vitro and in vivo.

3.637 Interaction of presenilins with FKBP38 promotes apoptosis by reducing mitochondrial Bcl-2

Wang, H-Q. et al
*Hum. Mol. Genet., **14(13)**, 1889-1902 (2005)*

Presenilins 1 and 2 (PS1/2), causative molecules for familial Alzheimer's disease (FAD), are multipass transmembrane proteins localized predominantly in the endoplasmic reticulum (ER) and Golgi apparatus. Heteromeric protein complexes containing PS1/2 are thought to participate in several functions, including intramembrane proteolysis mediated by their γ-secretase activities. Previous studies have shown that PS1/2 are also involved in the regulation of apoptotic cell death, although the underlying mechanism remains unknown. Here, we demonstrate that FKBP38, an immunophilin family member residing in the

mitochondrial membrane, is an authentic PS1/2-interacting protein. PS1/2 and FKBP38 form macromolecular complexes together with anti-apoptotic Bcl-2. PS1/2 promote the degradation of FKBP38 and Bcl-2 and sequester these proteins in the ER/Golgi compartments, thereby inhibiting FKBP38-mediated mitochondrial targeting of Bcl-2 via a γ-secretase-independent mechanism. Thus, PS1/2 increase the susceptibility to apoptosis by antagonizing the anti-apoptotic function of FKBP38. In contrast, C-terminal fragments of caspase-processed PS1/2 redistribute Bcl-2 to the mitochondria by abrogating the activity of full-length PS1/2, resulting in a dominant-negative anti-apoptotic effect. In cultured cells and mutant PS1-knockin mice brains, FAD-linked PS1/2 mutants enhance the pro-apoptotic activity by causing a more efficient reduction in mitochondrial Bcl-2 than wild-type PS1/2. These results suggest a novel molecular mechanism for the regulation of mitochondria-mediated apoptosis by competition between PS1/2 and FKBP38 for subcellular targeting of Bcl-2. Excessive pro-apoptotic activity of PS1/2 may play a role in the pathogenesis of FAD.

3.638 Analysis of proteome bound to D-loop region of mitochondrial DNA by DNA-linked affinity chromatography and reverse-phase liquid chromatography/tandem mass spectrometry
Choi, Y-S., Ryu, B-K., Min, H-K., Lee, S-W. and Pak, Y.K.
Ann. N.Y. Acad. Sci., ***1042***, *88-100 (2005)*

Mitochondrial dysfunction has been suggested as a causal factor for insulin resistance and diabetes. Previously we have shown a decrease of mitochondrial DNA (mtDNA) content in tissues of diabetic patients. The mitochondrial proteins, which regulate the mitochondrial biogensis, including transcription and replication of mtDNA, are encoded by nuclear DNA. Despite the potential function of the proteins bound to the D-loop region of mtDNA in regulating mtDNA transcription/replication, only a few proteins are known to bind the D-loop region of mtDNA. The functional association of these known proteins with insulin resistance is weak. In this study, we applied proteomic analysis to identify a group of proteins (proteome) that physically bind to D-loop DNA of mtDNA. We amplified D-loop DNA (1.1 kb) by PCR and conjugated the PCR fragments to CNBr-activated sepharose. Mitochondria fractions were isolated by both differential centrifugation and **Optiprep**-gradient ultracentrifugation. The D-loop DNA binding proteome fractions were enriched via this affinity chromatography and analyzed by SDS-PAGE. The proteins on the gel were transferred onto PVDF membrane and the peptide sequences of each band were subsequently analyzed by capillary reverse-phase liquid chromatography/tandem mass spectrometry (RPLC/MS/MS). We identified many D-loop DNA binding proteins, including mitochondrial transcription factor A (mtTFA, Tfam) and mitochondrial single-stranded DNA binding protein (mtSSBP) which were known to bind to mtDNA. We also report the possibility of novel D-loop binding proteins such as histone family proteins and high-mobility group proteins.

3.639 Intravesicular localization and exocytosis of α-synuclein and its aggregates
Lee, H.J., Patel, S. and Lee, S-J.
J. Neurosci., ***25(25)***, *6016-6024 (2005)*

α-Synuclein (α-syn), particularly in its aggregated forms, is implicated in the pathogenesis of Parkinson's disease and other related neurological disorders. However, the normal biology of α-syn and how it relates to the aggregation of the protein are not clearly understood. Because of the lack of the signal sequence and its predominant localization in the cytosol, α-syn is generally considered exclusively an intracellular protein. Contrary to this assumption, here, we show that a small percentage of newly synthesized α-syn is rapidly secreted from cells via unconventional, endoplasmic reticulum/Golgi-independent exocytosis. Consistent with this finding, we also demonstrate that a portion of cellular α-syn is present in the lumen of vesicles. Importantly, the intravesicular α-syn is more prone to aggregation than the cytosolic protein, and aggregated forms of α-syn are also secreted from cells. Furthermore, secretion of both monomeric and aggregated α-syn is elevated in response to proteasomal and mitochondrial dysfunction, cellular defects that are associated with Parkinson's pathogenesis. Thus, intravesicular localization and secretion are part of normal life cycle of α-syn and might also contribute to pathological function of this protein.

3.640 Analysis of human immunodeficiency virus type 1 Gag ubiquitination
Gottwein, E. And Kräusslich, H-G.
J. Virol., ***79(14)***, *9134-9144 (2005)*

Ubiquitin is important for the release of human immunodeficiency virus type 1 (HIV-1) and several other retroviruses, but the functional significance of Gag ubiquitination is unknown. To address this problem, we decided to analyze Gag ubiquitination in detail. A low percentage of the HIV-1 p6 protein has previously

been shown to be ubiquitinated, and published mutagenesis data suggested that Gag ubiquitination is largely lost upon mutation of the two lysine residues in p6. In this study, we show that Gag proteins lacking the p6 domain or the two lysine residues within p6 are ubiquitinated at levels comparable to those of the wild-type Gag protein. We detected monoubiquitinated forms of the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins in mature virus preparations. Protease digestion of Gag polyproteins extracted from immature virions indicated that ubiquitinated MA, CA, and possibly NC are as abundant as ubiquitinated p6. The HIV-1 late-domain motifs PTAP and LRSLF were not required for Gag ubiquitination, and mutation of the PTAP motif even resulted in an increase in the amount of Gag-Ub conjugates detected. Finally, at steady state, ubiquitinated Gag proteins were not enriched in either membrane-associated or virus-derived Gag fractions. In summary, these results indicate that HIV-1 Gag can be monoubiquitinated in all domains and that ubiquitination of lysine residues outside p6 may thus contribute to viral release and/or infectivity.

3.641 Hyperhomocysteinemia, a cardiac metabolic disease role of nitric oxide and the $p22^{phox}$ subunit of NADPH oxidase
Becker, J.S. et al
*Circulation, **111**, 2112-2118 (2005)*

***Background*—** Hyperhomocysteinemia (HHcy) is a reliable indicator of cardiovascular disease, in part because of the production of superoxide and scavenging of nitric oxide (NO). The present study assessed the impact of HHcy on the NO-dependent control of cardiac O_2 consumption and examined enzymatic sources of superoxide.
***Methods and Results*—** Rats and mice were fed methionine in drinking water for 5 to 9 weeks to increase plasma homocysteine, a process that did not cause significant changes in hemodynamic function. The ability of the NO agonists bradykinin and carbachol to reduce myocardial O_2 consumption in vitro was impaired by ≈40% in methionine-fed rats, and this impairment was proportional to their individual plasma homocysteine concentration. However, responses were restored in the presence of ascorbic acid, tempol, and apocynin, which inhibits NADPH oxidase assembly. Western blots showed no difference in Cu/Zn or Mn superoxide dismutase, endothelial NO synthase, or inducible NO synthase protein, but HHcy caused a 100% increase in the $p22^{phox}$ subunit of NADPH oxidase. Western blots with plasma membrane–enriched fractions of cell lysate detected elevated levels of $p22^{phox}$, $p67^{phox}$, and rac-1, which indicates increased oxidase assembly. Finally, mice lacking a functional $gp91^{phox}$ subunit of NADPH oxidase demonstrated normal NO-dependent regulation of myocardial O_2 consumption after methionine feeding.
***Conclusions*—** In HHcy, superoxide produced by NADPH oxidase reduces the ability of NO to regulate mitochondrial function in the myocardium. The severity of this effect is proportional to the increase in homocysteine.

3.642 Ceramide 1-phosphate, a mediator of phagocytosis
Hinkovska-Galcheva, V. Et al
*J. Biol. Chem., **280(28)**, 26612-26621 (2005)*

The agonist-stimulated metabolism of membrane lipids produces potent second messengers that regulate phagocytosis. We studied whether human ceramide kinase (hCERK) activity and ceramide 1-phosphate formation could lead to enhanced phagocytosis through a mechanism involving modulation of the membrane-structural order parameter. hCERK was stably transfected into COS-1 cells that were stably transfected with the $Fc\gamma$RIIA receptor. hCERK-transfected cells displayed a significant increase in phagocytic index in association with increased ceramide kinase activation and translocation to lipid rafts after activation with opsonized erythrocytes. When challenged with opsonized erythrocytes, hCERK-transfected cells increased phagocytosis by 1.5-fold compared with vector control and simultaneously increased ceramide 1-phosphate levels 2-fold compared with vector and unstimulated control cells. Control and hCERK-transfected cells were subjected to cellular fractionation. Utilizing an antibody against hCERK, we observed that CERK translocates during activation from the cytosol to a lipid raft fraction. The plasma membrane-structural order parameter of the transfectants was measured by labeling cells with Laurdan. Cells transfected with hCERK showed a higher liquid crystalline order than control cells with stimulation, conditions that are favorable for the promotion of membrane fusion at the sites of phagocytosis. The change in the structural order parameter of the lipid rafts probably contributes to phagocytosis by promoting phagosome formation.

3.643 Murine coronavirus requires lipid rafts for virus entry and cell-cell fusion but not for virus release
Choi, K.S., Aizaki, H. and Lai, M.M.

*J. Virol., **79(15)**, 9862-9871 (2005)*

Thorp and Gallagher first reported that depletion of cholesterol inhibited virus entry and cell-cell fusion of mouse hepatitis virus (MHV), suggesting the importance of lipid rafts in MHV replication (E. B. Thorp and T. M. Gallagher, J. Virol. **78:**2682-2692, 2004). However, the MHV receptor is not present in lipid rafts, and anchoring of the MHV receptor to lipid rafts did not enhance MHV infection; thus, the mechanism of lipid rafts involvement is not clear. In this study, we defined the mechanism and extent of lipid raft involvement in MHV replication. We showed that cholesterol depletion by methyl ß-cyclodextrin or filipin did not affect virus binding but reduced virus entry. Furthermore, MHV spike protein bound to nonraftraft membrane at 4°C but shifted to lipid rafts at 37°C, indicating a redistribution of membrane following virus binding. Thus, the lipid raft involvement in MHV entry occurs at a step following virus binding. We also found that the viral spike protein in the plasma membrane of the infected cells was associated with lipid rafts, whereas that in the Golgi membrane, where MHV matures, was not. Moreover, the buoyant density of the virion was not changed when MHV was produced from the cholesterol-depleted cells, suggesting that MHV does not incorporate lipid rafts into the virion. These results indicate that MHV release does not involve lipid rafts. However, MHV spike protein has an inherent ability to associate with lipid rafts. Correspondingly, cell-cell fusion induced by MHV was retarded by cholesterol depletion, consistent with the association of the spike protein with lipid rafts in the plasma membrane. These findings suggest that MHV entry requires specific interactions between the spike protein and lipid rafts, probably during the virus internalization step.

3.644 Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids
Pike, L.J., Han, X. and Gross, R.W.
*J. Biol. Chem., **280(29)**, 26796-26804*

The epidermal growth factor (EGF) receptor partitions into lipid rafts made using a detergent-free method, but is extracted from low density fractions by Triton X-100. By screening several detergents, we identified Brij 98 as a detergent in which the EGF receptor is retained in detergent-resistant membrane fractions. To identify the difference in lipid composition between those rafts that harbored the EGF receptor (detergent-free and Brij 98-resistant) and those that did not (Triton X-100-resistant), we used multidimensional electrospray ionization mass spectrometry to perform a lipidomics study on these three raft preparations. Although all three raft preparations were similarly enriched in cholesterol, the EGF receptor-containing rafts contained more ethanolamine glycerophospholipids and less sphingomyelin than did the non-EGF receptor-containing Triton X-100 rafts. As a result, the detergent-free and Brij 98-resistant rafts exhibited a balance of inner and outer leaflet lipids, whereas the Triton X-100 rafts contained a preponderance of outer leaflet lipids. Furthermore, in all raft preparations, the outer leaflet phospholipid species were significantly different from those in the bulk membrane, whereas the inner leaflet lipids were quite similar to those found in the bulk membrane. These findings indicate that the EGF receptor is retained only in rafts that exhibit a lipid distribution compatible with a bilayer structure and that the selection of phospholipids for inclusion into rafts occurs mainly on the outer leaflet lipids.

3.645 Light-dependent redistribution of arrestin in vertebrate rods is an energy-independent process governed by protein-protein interactions
Nair, K.S. et al
*Neuron, **46**, 555-567 (2005)*

In rod photoreceptors, arrestin localizes to the outer segment (OS) in the light and to the inner segment (IS) in the dark. Here, we demonstrate that redistribution of arrestin between these compartments can proceed in ATP-depleted photoreceptors. Translocation of transducin from the IS to the OS also does not require energy, but depletion of ATP or GTP inhibits its reverse movement. A sustained presence of activated rhodopsin is required for sequestering arrestin in the OS, and the rate of arrestin relocalization to the OS is determined by the amount and the phosphorylation status of photolyzed rhodopsin. Interaction of arrestin with microtubules is increased in the dark. Mutations that enhance arrestin-microtubule binding attenuate arrestin translocation to the OS. These results indicate that the distribution of arrestin in rods is controlled by its dynamic interactions with rhodopsin in the OS and microtubules in the IS and that its movement occurs by simple diffusion.

3.646 Role of fission yeast myosin I in organization of sterol-rich membrane domains
Takeda, T. and Chang, F.

*Current Biol., **15**, 1331-1336 (2005)*

Specialized membrane domains containing lipid rafts are thought to be important for membrane processes such as signaling and trafficking **[1 and 2].** An unconventional type I myosin has been shown to reside in lipid rafts and function to target a disaccharidase to rafts in brush borders of intestinal mammalian cells **[3].** In the fission yeast *Schizosaccharomyces pombe*, distinct sterol-rich membrane domains are formed at the cell division site and sites of polarized cell growth at cell tips **[4].** Here, we show that the sole *S. pombe* myosin I, myo1p, is required for proper organization of these membrane domains. *myo1* mutants lacking the TH1 domain exhibit a uniform distribution of sterol-rich membranes all over the plasma membrane throughout the cell cycle. These effects are independent of endocytosis because *myo1* mutants exhibit no endocytic defects. Conversely, overexpression of myo1p induces ectopic sterol-rich membrane domains. Myo1p localizes to nonmotile foci that cluster in sterol-rich plasma membrane domains and fractionates with detergent-resistant membranes. Because the myo1p TH1 domain may bind directly to acidic phospholipids, these findings suggest a model for how type I myosin contributes to the organization of specialized membrane domains.

3.647 Relationship between Alzheimer's disease clinical stage and Gq/11 in subcellular fractions of frontal cortex
Kelly, J.F. et al
*J. Neural Transm., **112**, 1049-1056 (2005)*

Alzheimer's disease (AD) is associated with impaired coupling of cell surface muscarinic cholinergic receptors to G proteins of the Gq/11 class in brain. This alteration may contribute to progression of cognitive impairment during the course of the disease. We hypothesized that increasing severity of cognitive impairment would be related to decreased levels of Gq/11 detected in key subcellular fractions made from postmortem brain tissue. In this study, we used Western blotting to determine the quantity of Gq/11α in P2, synaptic plasma membrane, cytoplasm, microsomal membrane, and lipid raft fractions prepared from superior frontal cortex gray matter of 25 patients with clinical AD confirmed by post-mortem examination. Multiple linear regression analysis that adjusted for age, sex, and education showed a linear relationship between frontal cortex synaptic plasma membrane Gq/11α levels and severity of cognitive impairment determined by Mini Mental State score measured proximate to death.

3.648 Two domains within the first putative transmembrane domain of presenilin 1 differentially influence presenilinase and γ-secretase activity
Brunkan, A.L. et al
*J. Neurochem., **94**, 1315-1328 (2005)*

Presenilins (PS) are thought to contain the active site for presenilinase endoproteolysis of PS and γ-secretase cleavage of substrates. The structural requirements for PS incorporation into the γ-secretase enzyme complex, complex stability and maturation, and appropriate presenilinase and γ-secretase activity are poorly understood. We used rescue assays to identify sequences in transmembrane domain one (TM1) of PS1 required to support presenilinase and γ-secretase activities. Swap mutations identified an N-terminal TM1 domain that is important for γ-secretase activity only and a C-terminal TM1 domain that is essential for both presenilinase and γ-secretase activities. Exchange of residues 95–98 of PS1 (sw95–98) completely abolishes both activities while the familial Alzheimer's disease mutation V96F significantly inhibits both activities. Reversion of residue 96 back to valine in the sw95–98 mutant rescues PS function, identifying V96 as the critical residue in this region. The TM1 mutants do not bind to an aspartyl protease transition state analog γ-secretase inhibitor, indicating a conformational change induced by the mutations that abrogates catalytic activity. TM1 mutant PS1 molecules retain the ability to interact with γ-secretase substrates and γ-secretase complex members, although Nicastrin stability is decreased by the presence of these mutants. γ-Secretase complexes that contain V96F mutant PS1 molecules display a partial loss of function for γ-secretase that alters the ratio of amyloid-β peptide species produced, leading to the amyloid-β peptide aggregation that causes familial Alzheimer's disease.

3.649 Effects of a mosquitocidal toxin on a mammalian epithelial cell line expressing its target receptor
Pauchet, Y. Et al
*Cell. Microbiol., **7(9)**, 1335-1344 (2005)*

The spread of diseases transmitted by *Anopheles* and *Culex* mosquitoes, such as malaria and West Nile fever, is a growing concern for human health. *Bacillus sphaericus* binary toxin (Bin) is one of the few

available bioinsecticides able to control populations of these mosquitoes efficiently. We previously showed that Bin binds to Cpm1, an α-glucosidase located on the apical side of *Culex* larval midgut epithelium. We analysed the effects of Bin by expressing a construct encoding Cpm1 in the mammalian epithelial MDCK cell line. Cpm1 is targeted to the apical side of polarized MDCK, where it is anchored by glycosylphosphatidylinositol (GPI) and displays α-glucosidase activity. Bin bound to transfected cells and induced a non-specific current presumably related to the opening of pores. The formation of these pores may be related to the location of the toxin/receptor complex in lipid raft microdomains. Finally, Bin promoted the time-dependent appearance of intracytoplasmic vacuoles but did not drive cell lysis. Thus, the dual functionality (enzyme/toxin receptor) of Cpm1 is fully conserved in MDCK cells and Cpm1 is an essential target protein for Bin cytotoxicity in *Culex* mosquitoes.

3.650 The golgin lava lamp mediates dynein-based Golgi movements during *Drosophila* cellularization
Papoulas, O., Hays, T.S. and Sisson, J.C.
*Nature Cell Biol., **7(8)**, 612-618 (2005)*

Drosophila melanogaster cellularization is a dramatic form of cytokinesis in which a membrane furrow simultaneously encapsulates thousands of cortical nuclei of the syncytial embryo to generate a polarized cell layer. Formation of this cleavage furrow depends on Golgi-based secretion and microtubules[1, 2, 3]. During cellularization, specific Golgi move along microtubules, first to sites of furrow formation and later to accumulate within the apical cytoplasm of the newly forming cells[3]. Here we show that Golgi movements and furrow formation depend on cytoplasmic dynein. Furthermore, we demonstrate that Lava lamp (Lva), a golgin protein that is required for cellularization, specifically associates with dynein, dynactin, cytoplasmic linker protein-190 (CLIP-190) and Golgi spectrin, and is required for the dynein-dependent targeting of the secretory machinery. The Lva domains that bind these microtubule-dependent motility factors inhibit Golgi movement and cellularization in a live embryo injection assay. Our results provide new evidence that golgins promote dynein-based motility of Golgi membranes.

3.651 Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol
Platta, H.W., Grunau, S., Rosenkrantz, K., Girzalsky, W. and Erdmann, R.
*Nature Cell Biol., **7(8)**, 817-822 (2005)*

Peroxisomal import receptors bind their cargo proteins in the cytosol and target them to docking and translocation machinery at the peroxisomal membrane (reviewed in ref. 1). The receptors release the cargo proteins into the peroxisomal lumen and, according to the model of cycling receptors, they are supposed to shuttle back to the cytosol. This shuttling of the receptors has been assigned to peroxins including the AAA peroxins Pex1p and Pex6p, as well as the ubiquitin-conjugating enzyme Pex4p (reviewed in ref. 2). One possible target for Pex4p is the PTS1 receptor Pex5p, which has recently been shown to be ubiquitinated[3, 4, 5]. Pex1p and Pex6p are both cytosolic and membrane-associated AAA ATPases of the peroxisomal protein import machinery, the exact function of which is still unknown. Here we demonstrate that the AAA peroxins mediate the ATP-dependent dislocation of the peroxisomal targeting signal-1 (PTS1) receptor from the peroxisomal membrane to the cytosol.

3.652 Exogenous nitric oxide reduces glucose transporters translocation and lactate production in ischemic myocardium in vivo
Lei, B. Et al
*PNAS, **102**, 6966-6971 (2005)*

Nitric oxide (NO) inhibits myocardial glucose transport and metabolism, although the underlying mechanism(s) and functional consequences of this effect are not clearly understood. We tested the hypothesis that NO inhibits the activation of AMP-activated protein kinase (AMPK) and translocation of cardiac glucose transporters (GLUTs; GLUT-4) and reduces lactate production. Ischemia was induced in open-chest dogs by a 66% flow reduction in the left anterior descending coronary artery (LAD). During ischemia, dogs were untreated (control) or treated by direct LAD infusion of (*i*) nitroglycerin (NTG) (0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$); (*ii*) 8-Br-cGMP (50 $\mu g \cdot kg^{-1} \cdot min^{-1}$); or (*iii*) NO synthase inhibitor L-nitro-argininemethylester (40 $\mu g \cdot kg^{-1} \cdot min^{-1}$; $n = 9$ per group). Cardiac substrate oxidation was measured with isotopic tracers. There were no differences in myocardial blood flow or oxygen delivery among groups; however, at 45 min of ischemia, the activation of AMPK was significantly less in NTG (77 ± 12% vs. nonischemic myocardium) and 8-Br-cGMP (104 ± 13%), compared with control (167 ± 17%). Similarly, GLUT-4 translocation was significantly reduced in NTG (74 ± 7%) and 8-Br-cGMP (120 ± 11%), compared with control (165 ± 17%). Glucose uptake and lactate output were 30% and 60% lower in NTG

compared with control. Inhibition of NO synthesis stimulated glucose oxidation (67% increase compared with control) but did not affect AMPK phosphorylation, GLUT-4 translocation and glucose uptake. Contractile function in the ischemic region was significantly improved by NTG and L-nitro-argininemethylester. In conclusion, in ischemic myocardium an NO donor inhibits glucose uptake and lactate production via a reduction in AMPK stimulation of GLUT-4 translocation, revealing a mechanism of metabolic modulation and myocardial protection activated by NO donors.

3.653 Role of membrane microdomains in PTH-mediated down-regulation of NaPi-Iia in opossum kidney cells

Nashiki, K. Et al

*Kidney Int., **68**, 1137-1147 (2005)*

Background. Parathyroid hormone (PTH) rapidly down-regulates type IIa sodium-dependent phosphate transporter (NaPi-IIa) via an endocytic pathway. Since the relationship between PTH signaling and NaPi-IIa endocytosis has not been explored, we investigated the role of membrane microdomains in this process.
Methods. We examined the submembrane localization of NaPi-IIa in opossum kidney (OK-N2) cells that stably expressed human NaPi-IIa, and searched for a PTH-induced specific phosphorylating substrate on their membrane microdomains by immunoblotting with specific antibody against phospho substrates of protein kinases.
Results. We found that NaPi-IIa was primarily localized in low-density membrane (LDM) domains of the plasma membrane; PTH reduced the levels of immunoreactive NaPi-IIa in these domains. Furthermore, PTH activated both protein kinase A (PKA) and protein kinase Cα (PKCa) and increased the phosphorylation of 250 kD and 80 kD substrates; this latter substrate was identified as ezrin, which a member of the ezrin-radixin-moesin (ERM) protein family. In response to PTH, ezrin was phosphorylated by both PKA and PKC. Dominant negative ezrin blocked the reduction in NaPi-IIa expression in the LDM domains that was induced by PTH.
Conclusion. These data suggest that NaPi-IIa and PTH-induced phosphorylated proteins that include ezrin are compartmentalized in LDM microdomains. This compartmentalization may play an important role in the down-regulation of NaPi-IIa via endocytosis.

3.654 B-cell receptor translocation to lipid rafts and associated signaling differ between prognostically important subgroups of chronic lymphocytic leukemia

Alsup, D.J. et al

*Cancer Res., **65(16)**, 7328-7337 (2005)*

Chronic lymphocytic leukemia (CLL) is a highly heterogeneous disease in which interaction of the malignant cells with antigen is thought to play a key role. Individual CLL-cell clones markedly differ in their ability to respond to B-cell receptor ligation, but the mechanism underlying the frequent hyporesponsiveness is incompletely understood. Our aim was to further clarify the extent and cause of the B-cell receptor signaling abnormality in CLL and to assign pathophysiologic relevance to the presence or absence of B-cell receptor responsiveness. We show that extracellular signal-regulated kinase-2 phosphorylation, intracellular Ca^{2+} increases, CD79a phosphorylation, and translocation of the B-cell receptor to lipid rafts in response to ligation with anti–immunoglobulin M (as a surrogate for antigen) are features of CLL cells with relatively unmutated *VH* genes (<5% deviation from germ line) and a poor prognosis. B-cell receptor stimulation in these cases also promoted cell survival. In clones with mutated *VH* genes (>5% deviation from germ line), surface immunoglobulin M ligation failed to induce receptor translocation to rafts or to prolong cell survival. This failure of receptor translocation observed in mutated CLL cells was associated with the constitutive exclusion of the B-cell receptor from rafts by a mechanism involving src-dependent interactions between the B-cell receptor and the actin cytoskeleton. We conclude that exposure to antigen promotes the survival of unmutated CLL clones, contributing to the poor prognosis of this group. In contrast, hyporesponsive mutated CLL clones may have developed into a stage where continuous exposure to antigen results in relative tolerance to antigenic stimulation mediated by the exclusion of the B-cell receptor from lipid rafts.

3.655 Overexpression of OSBP-related protein 2 (ORP2) induces changes in cellular cholesterol metabolism and enhances endocytosis

Hynynen, R. Et al

Biochem. J., 273-283 (2005)

ORP2 [OSBP (oxysterol-binding protein)-related protein 2] belongs to the 12-member mammalian ORP gene/protein family. We characterize in the present study the effects of inducible ORP2 overexpression on cellular cholesterol metabolism in HeLa cells and compare the results with those obtained for CHO cells (Chinese-hamster ovary cells) that express ORP2 constitutively. In both cell systems, the prominent phenotype is enhancement of [^{14}C]cholesterol efflux to all extracellular acceptors, which results in a reduction of cellular free cholesterol. No change was observed in the plasma membrane cholesterol content or distribution between raft and non-raft domains upon ORP2 expression. However, elevated HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase activity and LDL (low-density lipoprotein) receptor expression, as well as enhanced transport of newly synthesized cholesterol to a cyclodextrin-accessible pool, suggest that the ORP2 expression stimulates transport of cholesterol out of the endoplasmic reticulum. In contrast with ORP2/CHO cells, the inducible ORP2/HeLa cells do not show down-regulation of cholesterol esterification, suggesting that this effect represents an adaptive response to long-term cholesterol depletion in the CHO cell model. Finally, we provide evidence that ORP2 binds PtdIns(3,4,5)P_3 and enhances endocytosis, phenomena that are probably interconnected. Our results suggest a function of ORP2 in both cholesterol trafficking and control of endocytic membrane transport.

3.656 Phagocytic signaling molecules in lipid rafts of COS-1 cells transfected with FcγRIIA
Mansfield, P.J., Hinkovska-Galcheva, V., Borofsky, M.S., Shayman, J.A. and Boxer, L.A.
Biochem. Biophys. Res. Comm., ***331****, 132-138 (2005)*

COS-1 cells bearing FcγRIIA were used as a model to demonstrate co-localization of several enzymes previously shown to regulate neutrophil phagocytosis. In COS-1 cells, phospholipase D (PLD) in the membrane fraction was activated during phagocytosis. PLD was found almost exclusively in lipid rafts, along with RhoA and ARF1. Protein kinase C-δ (PKCδ) and Raf-1 translocated to lipid rafts. In neutrophils, ceramide levels increase during phagocytosis, indicating that FcγRIIA engagement initiates ceramide generation. Applying this model, we transfected COS-1 cells with FcγRIIA that had been mutated in the ITAM region, rendering them unable to ingest particles. When the mutant receptors were engaged, ceramide was generated and MAPK was activated normally, thus these processes did not require actual ingestion of particles. These results indicate that signaling proteins for phagocytosis are either constitutively present in, or are recruited to, lipid rafts where they are readily available to activate one another.

3.657 Caveolin-1 is essential for activation of Rac1 and NAD(P)H oxidase after angiotensin II type 1 receptor stimulation in vascular smooth muscle cells
Zuo, L. et al
Arterioscler. Thromb. Vasc. Biol., ***25****, 1824-1830 (2005)*

Objective— Angiotensin II (Ang II) is a potent mediator of vascular hypertrophy in vascular smooth muscle cells (VSMCs). These effects are mediated through the Ang II type 1 receptor (AT_1R) and require its trafficking through caveolin-1 (Cav1)–enriched lipid rafts and reactive oxygen species (ROS) derived from Rac1-dependent NAD(P)H oxidase. The specific role(s) of Cav1 in AT_1R signaling is incompletely understood.
Methods and Results— Knockdown of Cav1 protein by small interfering RNA (siRNA) inhibits Ang II–stimulated Rac1 activation and membrane translocation, H_2O_2 production, ROS-dependent epidermal growth factor receptor (EGF-R) transactivation, and subsequent phosphorylation of Akt without affecting ROS-independent extracellular signal-regulated kinase 1/2 phosphorylation. Ang II stimulates tyrosine phosphorylation of Sos-1, a Rac–guanine nucleotide exchange factor, which is inhibited by Cav1 siRNA, demonstrating involvement of Cav1 in Rac1 activation. Detergent-free fractionation showed that EGF-Rs are found basally in Cav1-enriched lipid raft membranes and associate with Cav1. Ang II stimulates AT_1R movement into these microdomains contemporaneously with the egress of EGF-R. Both aspects of this bidirectional receptor trafficking are inhibited by Cav1 siRNA. Moreover, Cav1 siRNA inhibits Ang II–induced vascular hypertrophy.
Conclusions— Cav1 plays an essential role in AT_1R targeting into Cav1-enriched lipid rafts and Rac1 activation, which are required for proper organization of ROS-dependent Ang II signaling linked to VSMC hypertrophy.
Angiotensin II (Ang II)–induced vascular hypertrophy is dependent on caveolae/lipid rafts and reactive oxygen species (ROS) derived from NAD(P)H oxidase. Using caveolin-1 siRNA, we demonstrate that caveolin-1 plays an essential role in AT_1 receptor targeting into caveolae/lipid rafts and Rac1 activation, which are required for ROS-dependent, growth-related Ang II signaling.

3.658 Maturation of Borma virus disease virus glycoprotein
Richt, J.A. and Garten, W.
*FEBS Lett., **579**, 4751-4756 (2005)*

The maturation of Borna disease virus (BDV) glycoprotein GP was studied in regard to intracellular compartmentalization, compartmentalization signal-domains, proteolytic processing, and packaging into virus particles. Our data show that BDV-GP is (i) predominantly located in the endoplasmic reticulum (ER), (ii) partially exists in the ER already as cleaved subunits GP-N and GP-C, (iii) is directed to the ER/cis-Golgi region by its transmembrane and/or cytoplasmic domains in CD8-BDV-GP hybrid constructs and (iv) is incorporated in the virus particles as authentic BDV glycoprotein exclusively in the cleaved form decorated with N-glycans of the complex type. Downregulation of BDV-glycoproteins on the cell surface, their limited proteolytic processing, and protection of antigenic epitopes on the viral glycoproteins by host-identical N-glycans are different strategies for persistent virus infections.

3.659 PAK5 kinase is an inhibitor of MARK/Par-1, which leads to stable microtubules and dynamic actin
Matenia, D. Et al
*Mol. Biol. Cell, **16**, 4410-4422 (2005)*

MARK/Par-1 is a kinase involved in development of embryonic polarity. In neurons, MARK phosphorylates tau protein and causes its detachment from microtubules, the tracks of axonal transport. Because the target sites of MARK on tau occur at an early stage of Alzheimer neurodegeneration, we searched for interaction partners of MARK. Here we report that MARK2 is negatively regulated by PAK5, a neuronal member of the p21-activated kinase family. PAK5 suppresses the activity of MARK2 toward its target, tau protein. The inhibition requires the binding between the PAK5 and MARK2 catalytic domains, but does not require phosphorylation. In transfected Chinese hamster ovary (CHO) cells both kinases show a vesicular distribution with partial colocalization on endosomes containing AP-1/2. Although MARK2 transfected alone destabilizes microtubules and stabilizes actin stress fibers, PAK5 keeps microtubules stable through the down-regulation of MARK2 but destabilizes the F-actin network so that stress fibers and focal adhesions disappear and cells develop filopodia. The results point to an inverse relationship between actin- and microtubule-related signaling by the PAK5 and MARK2 pathways that affect both cytoskeletal networks.

3.660 The novel fission yeast protein Pal1p interacts with Hip1-related Sla2p/End4p and is involved in cellular morphogenesis
Ge, W., Chew, T.G., Wachtler, V., Naqvi, S.N. and Balasubramanian, M.K.
*Mol. Biol. Cell, **16**, 4124-4138 (2005)*

The establishment and maintenance of characteristic cellular morphologies is a fundamental property of all cells. Here we describe *Schizosaccharomyces pombe* Pal1p, a protein important for maintenance of cylindrical cellular morphology. Pal1p is a novel membrane-associated protein that localizes to the growing tips of interphase cells and to the division site in cells undergoing cytokinesis in an F-actin- and microtubule-independent manner. Cells deleted for *pal1* display morphological defects, characterized by the occurrence of spherical and pear-shaped cells with an abnormal cell wall. Pal1p physically interacts and displays overlapping localization with the Huntingtin-interacting-protein (Hip1)-related protein Sla2p/End4p, which is also required for establishment of cylindrical cellular morphology. Sla2p is important for efficient localization of Pal1p to the sites of polarized growth and appears to function upstream of Pal1p. Interestingly, spherical *pal1*Δ mutants polarize to establish a pearlike morphology before mitosis in a manner dependent on the kelch-repeat protein Tea1p and the cell cycle inhibitory kinase Wee1p. Thus, overlapping mechanisms involving Pal1p, Tea1p, and Sla2p contribute to the establishment of cylindrical cellular morphology, which is important for proper spatial regulation of cytokinesis.

3.661 Membrane rafts segregate pro- from anti-apoptotic insulin-like growth factor-I receptor signaling in colon carcinoma cells stimulated by members of the tumor necrosis factor superfamily
Remacle-Bonnet, M. et al
*Am. J. Pathol., **167(3)**, 761-773 (2005)*

In the tumor microenvironment, autocrine/paracrine loops of insulin-like growth factors (IGFs) contribute to cancer cell survival. However, we report here that IGF-I can send contradictory signals that interfere with cell death induced by different ligands of the tumor necrosis factor (TNF) superfamily. IGF-I protected human colon carcinoma cells from TNF-α-induced apoptosis, but it enhanced the apoptotic

response to anti-Fas antibody and TNF-related apoptosis inducing ligand stimulation. This proapoptotic effect of IGF-I, observed in several but not all tested colon cancer cell lines, was mediated via the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway. Furthermore, IGF-I receptors (IGF-IR) were located in and out of membrane lipid rafts and were tyrosine autophosphorylated in response to IGF-I. However, disruption of rafts by acute cholesterol depletion shifted IGF-IR to non-raft domains, abolished the IGF-I-mediated proapoptotic effect, and inhibited the IGF-I-dependent IRS-1 and Akt recruitment into and phosphorylation/activation within lipid rafts. Replenishing cell membranes with cholesterol reversed these effects. Activation of extracellular-regulated kinase-1/2 and p38 mitogen-activated protein kinase, which convey the IGF-I anti-apoptotic effect, occurred independently of lipid rafts. Thus, we propose that segregation of IGF-IR in and out of lipid rafts may dynamically regulate the pro- and anti-apoptotic effects of IGF-I on apoptosis induced by TNF superfamily members.

3.662 The actin cytoskeleton differentially regulates platelet α-granule and dense-granule secretion
Flaumenahft, R. Et al
*Blood, **105(10)**, 3879-3887 (2005)*

Stimulation of platelets with strong agonists results in centralization of cytoplasmic organelles and secretion of granules. These observations have led to the supposition that cytoskeletal contraction facilitates granule release by promoting the interaction of granules with one another and with membranes of the open canalicular system. Yet, the influence of the actin cytoskeleton in controlling the membrane fusion events that mediate granule secretion remains largely unknown. To evaluate the role of the actin cytoskeleton in platelet granule secretion, we have assessed the effects of latrunculin A and cytochalasin E on granule secretion. Exposure of platelets to low concentrations of these reagents resulted in acceleration and augmentation of agonist-induced α-granule secretion with comparatively modest effects on dense granule secretion. In contrast, exposure of platelets to high concentrations of latrunculin A inhibited agonist-induced α-granule secretion but stimulated dense granule secretion. Incubation of permeabilized platelets with low concentrations of latrunculin A primed platelets for Ca^{2+}- or guanosine triphosphate (GTP)-γ-S-induced α-granule secretion. Latrunculin A-dependent α-granule secretion was inhibited by antibodies directed at vesicle-associated membrane protein (VAMP), demonstrating that latrunculin A supports soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein-dependent membrane fusion. These results indicate that the actin cytoskeleton interferes with platelet exocytosis and differentially regulates α-granule and dense granule secretion.

3.663 Plant and animal homeodomains use convergent mechanisms for intercellular transfer
Tasetto, M., Maizel, A., Osorio, J. and Joliot. A.
*EMBO Reports, **6(9)**, 885-890 (2005)*

Homeoproteins are defined by the structure of their DNA-binding domain, the homeodomain. Intercellular transfer of homeoprotein was observed *ex vivo* between animal cells and *in vivo* in higher plants. In the latter case, transfer is through intercytoplasmic channels that connect plant cells, but these do not exist in animals. Here, we show that the homeodomain of KNOTTED1, a maize homeoprotein, is transferred between animal cells and that a mutation in the homeodomain blocking the intercellular transfer of KNOTTED1 in plants also inhibits the transfer of the KNOTTED1 homeodomain in animal cells. This mutation decreases nuclear addressing, and its effect on nuclear import and intercellular transfer is reverted by the addition of an ectopic nuclear localization signal. We propose that, despite evolutionary distance and the differences in multicellular organization, similar mechanisms are at work for intercellular transfer of homeoprotein in plants and animals. Furthermore, our results suggest that, at least in animals, homeodomain secretion requires passage through the nucleus.

3.664 Purification of neuronal inclusions of patients with Huntington's disease reveals a broad range of N-terminal fragments of expanded huntingtin and insoluble polymers
Hoffner, G., Island, M-L. and Djian, P.
*J. Neurochem., **95**, 125-136 (2005)*

Huntington's disease resulting from huntingtin containing an expanded polyglutamine is associated with aggregates largely confined to neuronal inclusions, and with neuronal death. Inclusions are thought to originate from discrete N-terminal fragments of expanded huntingtin produced by specific endopeptidases. We have now purified the neuronal inclusions of Huntington's disease brain. When incubated in concentrated formic acid, purified inclusions release a polymer, an oligomer and a broad range of N-

terminal fragments of expanded huntingtin. The fragments and the polymeric forms are linked to each other by non-covalent bonds as they are both released by formic acid, whereas the polymeric forms themselves are presumably stabilized by covalent bonds, as they are resistant to formic acid. We also demonstrate the presence in affected areas of the brain but not in unaffected areas of a broad range of soluble N-terminal fragments of expanded huntingtin not yet associated with the inclusions and which are likely to be the precursors of the inclusions. Fragmentation of expanded huntingtin in Huntington's disease must result from the operation of multiple proteolytic activities with little specificity and not from that of a specific endopeptidase; subsequent aggregation of the fragments by covalent and non-covalent bonds leads to the formation of the inclusions.

3.665 Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein
Andersen, O.M. et al
*PNAS, **102(38)**, 13461-13466 (2005)*

sorLA (sorting protein-related receptor) is a type-1 membrane protein of unknown function that is expressed in neurons. Its homology to sorting receptors that shuttle between the plasma membrane, endosomes, and the Golgi suggests a related function in neuronal trafficking processes. Because expression of sorLA is reduced in the brain of patients with Alzheimer's disease (AD), we tested involvement of this receptor in intracellular transport and processing of the amyloid precursor protein (APP) to the amyloid β-peptide (Aβ), the principal component of senile plaques. We demonstrate that sorLA interacts with APP *in vitro* and in living cells and that both proteins colocalize in endosomal and Golgi compartments. Overexpression of sorLA in neurons causes redistribution of APP to the Golgi and decreased processing to Aβ, whereas ablation of sorLA expression in knockout mice results in increased levels of Aβ in the brain similar to the situation in AD patients. Thus, sorLA acts as a sorting receptor that protects APP from processing into Aβ and thereby reduces the burden of amyloidogenic peptide formation. Consequently, reduced receptor expression in the human brain may increase Aβ production and plaque formation and promote spontaneous AD.

3.666 Subcellular localization of iron regulatory proteins to Golgi and ER membranes
Patton, S.M., Pinero, D.J., Surguladze, N., Beard, J. and Connor, J.R.
*J. Cell Sci., **118**, 4365-4373 (2005)*

Interaction between iron regulatory proteins and iron responsive elements on certain mRNAs is at the core of regulation of intracellular iron homeostasis. Previous results suggested that in cultured cells iron regulatory proteins (IRPs) exist in cytosolic and microsomal subcellular locations and that this distribution is affected by cellular iron status. In this study, we tested the hypothesis that the membrane-associated fractions of iron regulatory proteins are specifically in the endoplasmic reticulum and Golgi membranes. Confocal microscopy revealed that IRP1 could be co-localized to the endoplasmic reticulum and the Golgi apparatus. To examine the intracellular distribution of IRPs biochemically, we used rats fed normal or iron-deficient diets. As expected, the IRPs were found predominantly in the cytosolic fraction. However, subfractionation of crude microsomal preparations revealed IRP1 in the Golgi apparatus. In animals fed an iron-deficient diet, IRP1 was found in the Golgi apparatus and the endoplasmic reticulum. To identify the mechanisms and factors involved in the localization of iron regulatory proteins in the cytosol and membrane fractions, cells were treated with a phorbol ester, a protein kinase C inhibitor (chelerythrine), hydrogen peroxide, interleukin-1ß, and 1,2-bis-(o-aminophenoxy)-ethane-*N,N,-N'N'*-tetraacetic acid tetraacetoxy-methyl ester. The results indicate that iron-regulatory-protein-binding activity in the membrane fraction can be altered by cell stress or iron status and that phosphorylation plays a role in the translocation. As a result of this study we propose a novel model for intracellular distribution of IRPs and identify differences between the two iron regulatory proteins.

3.667 LRP1B functions as a receptor for pseudomonas exotoxin
Pastrana, D.V., Hanson, A.J., Knisely, J., Bu, G. and FitzGerald, D.J.
*Biochim. Biophys. Acta, **1741**, 234-239 (2005)*

Pseudomonas aeruginosa is an opportunistic pathogen that produces several virulence factors, among them Pseudomonas Exotoxin A (PE). Previously, low-density lipoprotein receptor-related protein 1 (LRP1) was shown to be the primary receptor for PE. In this report, we show that a close family member, LRP1B, can also function as a receptor.

3.668 ***Helicobacter pylori*** **VacA cytotoxin: a probe for a clathrin-independent and Cdc42-dependent pinocytic pathway routed to late endosomes**
Gauthier, N.C. et al
*Mol. Biol. Cell, **16**, 4852-4866 (2005)*

The vacuolating cytotoxin VacA is a major virulence factor of *Helicobacter pylori*, a bacterium responsible for gastroduodenal ulcers and cancer. VacA associates with lipid rafts, is endocytosed, and reaches the late endocytic compartment where it induces vacuolation. We have investigated the endocytic and intracellular trafficking pathways used by VacA, in HeLa and gastric AGS cells. We report here that VacA was first bound to plasma-membrane domains localized above F-actin structures that were controlled by the Rac1 GTPase. VacA was subsequently pinocytosed by a clathrin-independent mechanism into cell peripheral early endocytic compartments lacking caveolin 1, the Rab5 effector early endosomes antigen-1 (EEA1) and transferrin. These compartments took up fluid-phase (as evidenced by the accumulation of fluorescent dextran) and glycosylphosphatidylinositol-anchored proteins (GPI-APs). VacA pinocytosis was controlled by Cdc42 and did not require cellular tyrosine kinases, dynamin 2, ADP-ribosylating factor 6, or RhoA GTPase activities. VacA was subsequently routed to EEA1-sorting endosomes and then sorted to late endosomes. During all these different endocytic steps, VacA was continuously associated with detergent resistant membrane domains. From these results we propose that VacA might be a valuable probe to study raft-associated molecules, pinocytosed by a clathrin-independent mechanism, and routed to the degradative compartment.

3.669 **Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by SIP_1 receptor, PI3 kinase, Tiam1/Rac1, and α-actinin**
Singleton, P.A., Dudek, S.M., Chiang, E.T. and Garcia, J.G.N.
*FASEB J., **19**, 1646-1656 (2005)*

Endothelial cell (EC) barrier dysfunction results in increased vascular permeability observed in inflammation, tumor angiogenesis, and atherosclerosis. The platelet-derived phospholipid sphingosine-1-phosphate (S1P) decreases EC permeability in vitro and in vivo and thus has obvious therapeutic potential. We examined S1P-mediated human pulmonary artery EC signaling and barrier regulation in caveolin-enriched microdomains (CEM). Immunoblotting from S1P-treated EC revealed S1P-mediated rapid recruitment (1 μM, 5 min) to CEMs of the S1P receptors $S1P_1$ and $S1P_3$, p110 PI3 kinase α and ß catalytic subunits, the Rac1 GEF, Tiam1, and α-actinin isoforms 1 and 4. Immunoprecipitated p110 PI3 kinase catalytic subunits from S1P-treated EC exhibited PIP_3 production in CEMs. Immunoprecipitation of S1P receptors from CEM fractions revealed complexes containing Tiam1 and $S1P_1$. PI3 kinase inhibition (LY294002) attenuated S1P-induced Tiam1 association with $S1P_1$, Tiam1/Rac1 activation, α-actinin-1/4 recruitment, and EC barrier enhancement. Silencing of either $S1P_1$ or Tiam1 expression resulted in the loss of S1P-mediated Rac1 activation and α-actinin-1/4 recruitment to CEM. Finally, silencing $S1P_1$, Tiam1, or both α-actinin isoforms 1/4 inhibits S1P-induced cortical F-actin rearrangement and S1P-mediated barrier enhancement. Taken together, these results suggest that S1P-induced recruitment of $S1P_1$ to CEM fractions promotes PI3 kinase-mediated Tiam1/Rac1 activation required for α-actinin-1/4-regulated cortical actin rearrangement and EC barrier enhancement.—Singleton, P. A., Dudek, S. M., Chiang, E. T., Garcia, J. G. N. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by $S1P_1$ receptor, PI3 kinase, Tiam1/Rac1 and α-actinin.

3.670 **Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission**
Gromley, A. et al
*Cell, **123**, 75-87 (2005)*

The terminal step in cytokinesis, called abscission, requires resolution of the membrane connection between two prospective daughter cells. Our previous studies demonstrated that the coiled-coil protein centriolin localized to the midbody during cytokinesis and was required for abscission. Here we show that centriolin interacts with proteins of vesicle-targeting exocyst complexes and vesicle-fusion SNARE complexes. These complexes require centriolin for localization to a unique midbody-ring structure, and disruption of either complex inhibits abscission. Exocyst disruption induces accumulation of v-SNARE-containing vesicles at the midbody ring. In control cells, these v-SNARE vesicles colocalize with a GFP-

tagged secreted polypeptide. The vesicles move to the midbody ring asymmetrically from one prospective daughter cell; the GFP signal is rapidly lost, suggesting membrane fusion; and subsequently the cell cleaves at the site of vesicle delivery/fusion. We propose that centriolin anchors protein complexes required for vesicle targeting and fusion and integrates membrane-vesicle fusion with abscission.

3.671 Identification of AQP5 in lipid rafts and its translocation to apical membranes by activation of M_3 mAChRs in interlobular ducts of rat parotid gland
Ishikawa, Y. Et al
*Am. J. Physiol Cell Physiol., **289**, C1303-C1311 (2005)*

Aquaporin-5 (AQP5), an apical plasma membrane (APM) water channel in salivary glands, lacrimal glands, and airway epithelium, has an important role in fluid secretion. M_3 muscarinic acetylcholine receptor (mAChR)-induced changes in AQP5 localization in rat parotid glands were investigated with immunofluorescence or immunoelectron microscopy, detergent solubility, and gradient density floatation assays. Confocal microscopy revealed AQP5 localization in intracellular vesicles of interlobular duct cells in rat parotid glands and AQP5 trafficking to the APM 10 min after injection of the mAChR agonist cevimeline. Conversely, 60 min after injection, there was a diffuse pattern of AQP5 staining in the cell cytoplasm. The calcium ionophore A-23187 mimicked the effects of cevimeline. Immunoelectron microscopic studies confirmed that cevimeline induced AQP5 trafficking from intracellular structures to APMs in the interlobular duct cells of rat parotid glands. Lipid raft markers flotillin-2 and GM1 colocalized with AQP5 and moved with AQP5 in response to cevimeline. Under control conditions, the majority of AQP5 localized in the Triton X-100-insoluble fraction and floated to the light-density fraction on discontinuous density gradients. After 10-min incubation of parotid tissue slices with cevimeline or A-23187, AQP5 levels decreased in the Triton X-100-insoluble fraction and increased in the Triton X-100-soluble fraction. Thus AQP5 localizes in the intracellular lipid rafts, and M_3 mAChR activation induces AQP5 trafficking to the APM with lipid rafts via intracellular Ca^{2+} signaling and induces AQP5 dissociation from lipid rafts to nonrafts on the APM in the interlobular duct cells of rat parotid glands.

3.672 Loss- and gain-of-function analysis of the lipid raft proteins *reggie/flotillin* in *Drosphilia*: they are posttranslationally regulated, and misexpression interferes with wing and eye development
Hoehne, M., de Couet, H.G., Stuermer, C.A.O. and Fischbach, K-F.
*Mol. Cell. Neurosci., **30**, 326-338 (2005)*

Reggie/Flotillin proteins are upregulated after optic nerve dissection and evolutionary highly conserved components of lipid rafts. Whereas many biochemical and cell culture studies suggest an involvement in the assembly of multiprotein complexes at cell contact sites, not much is known about their biological in vivo functions. We therefore set out to study the expression pattern and the effects of loss- and gain-of-function in the *Drosophila melanogaster* model system. We found that in flies these proteins are mainly expressed in axons at the root of fiber tracts, in places where strong fasciculation is required, e.g. at the neck of the peduncle of the mushroom bodies and in the optic chiasms. Despite their evolutionary conservation which implies fundamental and important functions, a P-element-induced null mutant (KG00210) of *reggie1/flotillin2* (*reggie1/flo2)* in *D. melanogaster* shows no apparent phenotypic defects. This was even more surprising as we show that in this *reggie1/flo2* null mutant the paralogous Reggie2/Flo1 protein is unstable and degraded, while the transcript is still present. The requirement of Reggie1/Flo2 for Reggie2/Flo1 stabilization is confirmed by misexpression experiments. Reggie2/Flo1 can only be misexpressed when Reggie1/Flo2 is provided as well. Conversely, Reggie1/Flo2 immunoreactivity can be detected, when its transgene is misexpressed alone. Using appropriate Gal4 driver lines, misexpression of Reggie1/Flo2 alone or together with Reggie2/Flo1 in the eye imaginal disc results in a specific and severe mislocalization of cell adhesion molecules of the immunoglobulin superfamily (IgCAMs) (while DE-Cadherin is unaffected) and in differentiation defects pointing to impaired signaling. In the wing imaginal disc, global overexpression of Reggie/Flotillin proteins leads to a significant extension of the Wingless signal and severely disrupts normal wing development. Our data support the notion that Reggie/Flotillin proteins are implicated in signaling processes at cellular contact sites.

3.673 Comparative genomic analysis reveals a novel mitochondrial isoform of human rTS protein and unusual phylogenetic distribution of the rTS gene
Liang, P., Nair, J.R., Song, L., McGuire, J.J. and Dolnick, B.J.
*BMC Genomics, **6**, 125 (2005)*

Background

The rTS gene (*ENOSF1*), first identified in *Homo sapiens* as a gene complementary to the thymidylate synthase (*TYMS*) mRNA, is known to encode two protein isoforms, rTSα and rTSβ. The rTSβ isoform appears to be an enzyme responsible for the synthesis of signaling molecules involved in the down-regulation of thymidylate synthase, but the exact cellular functions of rTS genes are largely unknown.
Results
Through comparative genomic sequence analysis, we predicted the existence of a novel protein isoform, rTS, which has a 27 residue longer N-terminus by virtue of utilizing an alternative start codon located upstream of the start codon in rTSβ. We observed that a similar extended N-terminus could be predicted in all rTS genes for which genomic sequences are available and the extended regions are conserved from bacteria to human. Therefore, we reasoned that the protein with the extended N-terminus might represent an ancestral form of the rTS protein. Sequence analysis strongly predicts a mitochondrial signal sequence in the extended N-terminal of human rTSγ, which is absent in rTSβ. We confirmed the existence of rTS in human mitochondria experimentally by demonstrating the presence of both rTSγ and rTSβ proteins in mitochondria isolated by subcellular fractionation. In addition, our comprehensive analysis of rTS orthologous sequences reveals an unusual phylogenetic distribution of this gene, which suggests the occurrence of one or more horizontal gene transfer events.
Conclusion
The presence of two rTS isoforms in mitochondria suggests that the rTS signaling pathway may be active within mitochondria. Our report also presents an example of identifying novel protein isoforms and for improving gene annotation through comparative genomic analysis.

3.674 Brome mosaic virus 1a nucleoside triphosphatase/helicase domain plays crucial roles in recruiting RNA replication templates
Wang, X. et al
J. Virol., ***79(21)****, 13747-13758 (2005)*

Positive-strand RNA virus RNA replication is invariably membrane associated and frequently involves viral proteins with nucleoside triphosphatase (NTPase)/helicase motifs or activities. Brome mosaic virus (BMV) encodes two RNA replication factors: 1a has a C-terminal NTPase/helicase-like domain, and $2a^{pol}$ has a central polymerase domain. 1a accumulates on endoplasmic reticulum membranes, recruits $2a^{pol}$, and induces 50- to 70-nm membrane invaginations (spherules) serving as RNA replication compartments. 1a also recruits BMV replication templates such as genomic RNA3. In the absence of $2a^{pol}$, 1a dramatically stabilizes RNA3 by transferring RNA3 to a membrane-associated, nuclease-resistant state that appears to correspond to the interior of the 1a-induced spherules. Prior results show that the 1a NTPase/helicase-like domain contributes to RNA recruitment. Here, we tested mutations in the conserved helicase motifs of 1a to further define the roles of this domain in RNA template recruitment. All 1a helicase mutations tested showed normal 1a accumulation, localization to perinuclear endoplasmic reticulum membranes, and recruitment of $2a^{pol}$. Most 1a helicase mutants also supported normal spherule formation. Nevertheless, these mutations severely inhibited RNA replication and 1a-induced stabilization of RNA3 in vivo. For such 1a mutants, the membrane-associated RNA3 pool was both reduced and highly susceptible to added nuclease. Thus, 1a recruitment of viral RNA templates to a membrane-associated, nuclease-resistant state requires additional functions beyond forming spherules and recruiting RNA to membranes, and these functions depend on the 1a helicase motifs. The possibility that, similar to some double-stranded RNA viruses, the 1a NTPase/helicase-like domain may be involved in importing viral RNAs into a preformed replication compartment is discussed.

3.675 Nef is physically recruited into the immunological synapse and potentiates T cell activation early after TCR engagemant
Fenard, D. et al
J. Immunol., ***175****, 6050-6057 (2005)*

The HIV-1 protein Nef enhances viral pathogenicity and accelerates disease progression in vivo. Nef potentiates T cell activation by an unknown mechanism, probably by optimizing the intracellular environment for HIV replication. Using a new T cell reporter system, we have found that Nef more than doubles the number of cells expressing the transcription factors NF-κB and NFAT after TCR stimulation. This Nef-induced priming of TCR signaling pathways occurred independently of calcium signaling and involved a very proximal step before protein kinase C activation. Engagement of the TCR by MHC-bound Ag triggers the formation of the immunological synapse by recruiting detergent-resistant membrane microdomains, termed lipid rafts. Approximately 5–10% of the total cellular pool of Nef is localized within lipid rafts. Using confocal and real-time microscopy, we found that Nef in lipid rafts was recruited into the

immunological synapse within minutes after Ab engagement of the TCR/CD3 and CD28 receptors. This recruitment was dependent on the N-terminal domain of Nef encompassing its myristoylation. Nef did not increase the number of cell surface lipid rafts or immunological synapses. Recently, studies have shown a specific interaction of Nef with an active subpopulation of p21-activated kinase-2 found only in the lipid rafts. Thus, the corecruitment of Nef and key cellular partners (e.g., activated p21-activated kinase-2) into the immunological synapse may underlie the increased frequency of cells expressing transcriptionally active forms of NF-κB and NFAT and the resultant changes in T cell activation.

3.676 γ-Secretase is a functional component of phagosomes
Jutras, I. et al
J. Biol. Chem., ***280(43)***, *36310-36317 (2005)*

γ-Secretase is a high molecular mass protein complex that catalyzes the intramembrane cleavage of its protein substrates. Two proteins involved in phagocytosis, CD44 and the low density lipoprotein receptor-related protein, are γ-secretase substrates, suggesting that this complex might regulate some aspects of phagocytosis. Our results indicate that the four components of γ-secretase, *viz.* presenilin, nicastrin, APH-1, and PEN-2, are present and enriched on phagosome membranes from both murine macrophages and *Drosophila* S2 phagocytes. The γ-secretase components form high molecular mass complexes in lipid microdomains of the phagosome membrane with the topology expected for the functional enzyme. In contrast to the majority of the phagosome proteins studied so far, which appear to associate transiently with this organelle, γ-secretase resides on newly formed phagosomes and remains associated throughout their maturation into phagolysosomes. Finally, our results indicate that interferon-γ stimulates γ-secretase-dependent cleavages on phagosomes and that γ-secretase activity may be involved in the phagocytic response of macrophages to inflammatory cytokines.

3.677 The blood-brain barrier transmigrating single domain antibody: mechanism of transport and antigenic epitopes in human brain endothelial cells
Abulrop, A., Sprong, H., Van Bergen en Henegouwen P. and Stanimirovic, D.
J. Neurochem., ***95***, *1201-1214 (2005)*

Antibodies against receptors that undergo transcytosis across the blood–brain barrier (BBB) have been used as vectors to target drugs or therapeutic peptides into the brain. We have recently discovered a novel single domain antibody, FC5, which transmigrates across human cerebral endothelial cells *in vitro* and the BBB *in vivo*. The purpose of this study was to characterize mechanisms of FC5 endocytosis and transcytosis across the BBB and its putative receptor on human brain endothelial cells. The transport of FC5 across human brain endothelial cells was polarized, charge independent and temperature dependent, suggesting a receptor-mediated process. FC5 taken up by human brain endothelial cells co-localized with clathrin but not with caveolin-1 by immunochemistry and was detected in clathrin-enriched subcellular fractions by western blot. The transendothelial migration of FC5 was reduced by inhibitors of clathrin-mediated endocytosis, K^+ depletion and chlorpromazine, but was insensitive to caveolae inhibitors, filipin, nystatin or methyl-β-cyclodextrin. Following internalization, FC5 was targeted to early endosomes, bypassed late endosomes/lysosomes and remained intact after transcytosis. The transcytosis process was inhibited by agents that affect actin cytoskeleton or intracellular signaling through PI3-kinase. Pretreatment of human brain endothelial cells with wheatgerm agglutinin, sialic acid, α(2,3)-neuraminidase or *Maackia amurensis* agglutinin that recognizes α(2,3)-, but not with *Sambucus nigra* agglutinin that recognizes α(2,6) sialylgalactosyl residues, significantly reduced FC5 transcytosis. FC5 failed to recognize brain endothelial cells-derived lipids, suggesting that it binds luminal α(2,3)-sialoglycoprotein receptor which triggers clathrin-mediated endocytosis. This putative receptor may be a new target for developing brain-targeting drug delivery vectors.

3.678 Lipids as modulators of proteolytic activity of BACE. Involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro
Kalvodova, L. et al
J. Biol. Chem., ***280(44)***, *36815-36823 (2005)*

The β-secretase, BACE, is a membrane spanning aspartic protease, which cleaves the amyloid precursor protein (APP) in the first step of proteolytic processing leading to the formation of the neurotoxic β-amyloid peptide (Aβ). Previous results have suggested that the regulation of β-secretase and BACE access to APP is lipid dependent, and involves lipid rafts. Using the baculovirus expression system, we have

expressed recombinant human full-length BACE in insect cells and purified milligram amounts to homogeneity. We have studied partitioning of fluorophor-conjugated BACE between the liquid ordered and disordered phases in giant (10–150 μm) unilamellar vesicles, and found ~20% to associate with the raft-like, liquid-ordered phase; the fraction associated with liquid-ordered phase increased upon cross-linking of raft lipids. To examine involvement of individual lipid species in modulating BACE activity, we have reconstituted the purified BACE in large (~100 nm) unilamellar vesicles, and determined its specific activity in vesicles of various lipid compositions. We have identified 3 groups of lipids that stimulate proteolytic activity of BACE: 1) neutral glycosphingolipids (cerebrosides), 2) anionic glycerophospholipids, and 3) sterols (cholesterol).

3.679 Cholesterol-dependent syntaxin-4 and SNAP-23 clustering regulates caveolar fusion with the endothelial plasma
Predescu, S.A., Predescu,, D.N., Shimizu, K., Klein, I.K. and Malik, A.B.
J. Biol. Chem., ***280(44)***, *37130-37138 (2005)*

We determined the organization of target (t) SNARE proteins on the basolateral endothelial plasma membrane (PM) and their role in the mechanism of caveolar fusion. Studies were performed in a cell-free system involving endothelial PM sheets and isolated biotin-labeled caveolae. We monitored the fusion of caveolae with the PM by the detection of biotin-streptavidin complexes using correlative high resolution fluorescence microscopy and gold labeling electron microscopy on ultrathin sections of PM sheets. Imaging of PM sheets demonstrated and biochemical findings showed that the t-SNARE proteins present in endothelial cells (SNAP-23 and syntaxin-4) formed cholesterol-dependent clusters in discrete areas of the PM. Upon fusion of caveolae with the target PM, 50% of the caveolae co-localized with the t-SNARE clusters, indicating that these caveolae were at the peak of the fusion reaction. Fluorescent streptavidin staining of PM sheets correlated with the ultrastructure in the same area. These findings demonstrate that t-SNARE clusters in the endothelial target PM serve as the fusion sites for caveolae during exocytosis.

3.680 Association of human immunodeficiency virus type 1 Gag with membrane does not require highly basic sequences in the nucleocapsid: use of a novel Gag multimerization assay
Ono, A., Waheed, A.A., Joshi, A.a and Freed, E.O.
J. Virol., ***79(22)***, *14131-14140 (2005)*

Human immunodeficiency virus type 1 (HIV-1) particle production, a process driven by the Gag polyprotein precursor, occurs on the plasma membrane in most cell types. The plasma membrane contains cholesterol-enriched microdomains termed lipid rafts, which can be isolated as detergent-resistant membrane (DRM). Previously, we and others demonstrated that HIV-1 Gag is associated with DRM and that disruption of Gag-raft interactions impairs HIV-1 particle production. However, the determinants of Gag-raft association remain undefined. In this study, we developed a novel epitope-based Gag multimerization assay to examine whether Gag assembly is essential for its association with lipid rafts. We observed that membrane-associated, full-length Gag is poorly detected by immunoprecipitation relative to non-membrane-bound Gag. This poor detection is due to assembly-driven masking of Gag epitopes, as denaturation greatly improves immunoprecipitation. Gag mutants lacking the Gag-Gag interaction domain located in the N terminus of the nucleocapsid (NC) were efficiently immunoprecipitated without denaturation, indicating that the epitope masking is caused by higher-order Gag multimerization. We used this assay to examine the relationship between Gag assembly and Gag binding to total cellular membrane and DRM. Importantly, a multimerization-defective NC mutant displayed wild-type levels of membrane binding and DRM association, indicating that NC-mediated Gag multimerization is dispensable for association of Gag with membrane or DRM. We also demonstrate that different properties of sucrose and **iodixanol** membrane flotation gradients may explain some discrepancies regarding Gag-raft interactions. This report offers new insights into the association of HIV-1 Gag with membrane and with lipid rafts.

3.681 A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells
Koch, A., Yoon, Y., Bonekamp, N.A., McNiven, M.A. and Schrader, M.
Mol. Biol. Cell, ***16***, *5077-5086 (2005)*

The mammalian dynamin-like protein DLP1/Drp1 has been shown to mediate both mitochondrial and peroxisomal fission. In this study, we have examined whether hFis1, a mammalian homologue of yeast Fis1, which has been shown to participate in mitochondrial fission by an interaction with DLP1/Drp1, is also involved in peroxisomal growth and division. We show that hFis1 localizes to peroxisomes in addition to mitochondria. Through differential tagging and deletion experiments, we demonstrate that the

transmembrane domain and the short C-terminal tail of hFis1 is both necessary and sufficient for its targeting to peroxisomes and mitochondria, whereas the N-terminal region is required for organelle fission. hFis1 promotes peroxisome division upon ectopic expression, whereas silencing of Fis1 by small interfering RNA inhibited fission and caused tubulation of peroxisomes. These findings provide the first evidence for a role of Fis1 in peroxisomal fission and suggest that the fission machinery of mitochondria and peroxisomes shares common components.

3.682 GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment
Augustin, R., Riley, J. and Moley, K.H.
*Traffic, **6**, 1196-1212 (2005)*

Glucose transporter 8 (GLUT8) contains a cytoplasmic N-terminal dileucine motif and localizes to a thus far unidentified intracellular compartment. Translocation of GLUT8 to the plasma membrane (PM) was found in insulin-treated mouse blastocysts. Using overexpression of GLUT8 in adipocytes and neuronal cells however, insulin treatment or depolarization of the cells did not lead to GLUT8 PM translocation in other studies. In addition, other experiments showing dynamin-dependent endocytosis of GLUT8 suggested that GLUT8 recycles between an endosomal compartment and the PM. To reveal the functional/physiological role of GLUT8, we studied its subcellular localization in 3T3L1, HEK293 and CHO cells. We show that GLUT8 does not co-localize with GLUT4 and does not redistribute to the PM after treatment with insulin, ionophores or okadaic acid in these cell lines. Once endocytosed, GLUT8 does not recycle to the PM. GLUT8 localizes to late endosomes and lysosomes. An interspecies GLUT8 - sequence alignment revealed the presence of a highly conserved late endosomal/lysosomal-targeting motif ([DE]XXXL[LI]). Changing the glutamate to arginine as found in GLUT4 (RRXXXLL) alters GLUT8 endocytosis and retains the transporter at the PM. Furthermore, sorting GLUT8 to late endosomes/lysosomes does not require prior presence of GLUT8 at the PM followed by its endocytosis. In summary, GLUT8 does not reside in a recycling vesicle pool and is distinct from GLUT4. From our data, we postulate a role for GLUT8 in transport of hexoses across intracellular membranes, for example in specific compartments of GLUT8 expression such as the acrosome of mature spermatozoa or secretory granules in neurons. Furthermore, a role for GLUT8 in hexose transport across the lysosomal membrane, a transport mechanism that has long been suggested but unexplained, is discussed.

3.683 The VirE1VirE2 complex of *Agrobacterium tumefaciens* interacts with single-stranded DNA and forms channels
Duckely, M. et al
*Mol. Microbiol., **58(4)**, 1130-1142 (2005)*

The VirE2 protein is crucial for the transfer of single-stranded DNA (ssDNA) from *Agrobacterium tumefaciens* to the nucleus of the plant host cell because of its ssDNA binding activity, assistance in nuclear import and putative ssDNA channel activity. The native form of VirE2 in *Agrobacterium*'s cytoplasm is in complex with its specific chaperone, VirE1. Here, we describe the ability of the VirE1VirE2 complex to both bind ssDNA and form channels. The affinity of the VirE1VirE2 complex for ssDNA is slightly reduced compared with VirE2, but the kinetics of binding to ssDNA are unaffected by the presence of VirE1. Upon binding of VirE1VirE2 to ssDNA, similar helical structures to those reported for the VirE2─ssDNA complex were observed by electron microscopy. The VirE1VirE2 complex can release VirE1 once the VirE2─ssDNA complexes assembled. VirE2 exhibits a low affinity for small unilamellar vesicles composed of bacterial lipids and a high affinity for lipid vesicles containing sterols and sphingolipids, typical components of animal and plant membranes. In contrast, the VirE1VirE2 complex associated similarly with all kind of lipids. Finally, black lipid membrane experiments revealed the ability of the VirE1VirE2 complex to form channels. However, the majority of the channels displayed a conductance that was a third of the conductance of VirE2 channels. Our results demonstrate that the binding of VirE1 to VirE2 does not inhibit VirE2 functions and that the effector─chaperone complex is multifunctional.

3.684 Biochemical characterization of intracellular membranes bearing Trk neurotrophin receptors
Yano, H. and Chao, M.V.
*Neurochem. Res., **30(6/7)**, 767-777 (2005)*

Neurotrophin receptor trafficking plays an important role in directing cellular communication in developing as well as mature neurons. However, little is known about the requirements for intracellular

localization of the neurotrophin receptors in neurons. To isolate the subcellular membrane compartments containing the Trk neurotrophin receptor, we performed biochemical subcellular fractionation experiments using primary cortical neurons and rat PC12 pheochromocytoma cells. By differential centrifugation and density gradient centrifugation, we have isolated Trk-bearing compartments, suggesting distinct membranous localization of Trk receptors. A number of Trk-interacting proteins, such as GIPC and dynein light chain Tctex-1 were found in these fractions. Additionally, membranes enriched in phosphorylated activated forms of Trk receptors were found upon ligand treatment in primary neurons and PC12 cells. Interestingly, density gradient centrifugation experiments showed that Trk receptors from PC12 cells are present in heavy membrane fractions, while Trk from primary neurons are fractionated in lighter membrane fractions. These results suggest that the intracellular membrane localization of Trk can differ according to cell type. Taken together, these biochemical approaches allowed separation of distinct Trk-bearing membrane pools, which may be involved in different functions of neurotrophin receptor signaling and trafficking.

3.685 Cytotoxicity of an anti-cancer lysophospholipid through selective modification of lipid raft composition

Zaremberg, V., Gajate, C., Cacharro, L.M., Mollinedo, F. and McMaster, C.R.
J. Biol. Chem., ***280(45)***, *38047-38058 (2005)*

Edelfosine is a prototypical member of the alkylphosphocholine class of antitumor drugs. *Saccharomyces cerevisiae* was used to screen for genes that modulate edelfosine cytotoxicity and identified sterol and sphingolipid pathways as relevant regulators. Edelfosine addition to yeast resulted in the selective partitioning of the essential plasma membrane protein Pma1p out of lipid rafts. Microscopic analysis revealed that Pma1p moved from the plasma membrane to intracellular punctate regions and finally localized to the vacuole. Consistent with altered sterol and sphingolipid synthesis resulting in increased edelfosine sensitivity, mislocalization of Pma1p was preceded by the movement of sterols out of the plasma membrane. Cells with enfeebled endocytosis and vacuolar protease activities prevented edelfosine-mediated (i) mobilization of sterols, (ii) loss of Pma1p from lipid rafts, and (iii) cell death. The activities of proteins and signaling processes are meaningfully altered by changes in lipid raft biophysical properties. This study points to a novel mode of action for an anti-cancer drug through modification of plasma membrane lipid composition resulting in the displacement of an essential protein from lipid rafts.

3.686 Thermally induced changes in lipid composition of raft and non-raft regions of hepatocyte plasma membranes of rainbow trout

Zehmer, J.K. and Hazel, J.R.
J. Exp. Biol., ***208***, *4283-4290 (2005)*

In poikilotherms, increases in plasma membrane (PM) cholesterol and an increase in the degree of lipid acyl chain saturation commonly accompany an increase in growth temperature. This has typically been interpreted in terms of membrane fluidity/order homeostasis, but these changes would also be expected to stabilize the structure of PM rafts against thermal perturbation. Rafts are microdomains that organize the molecules of many signaling cascades and are formed as a result of interactions between lipids with saturated acyl chains and cholesterol. No study to date has examined the thermally induced compositional changes of raft and non-raft regions of the PM separately. In this study we have measured the phospholipid class composition and fatty acid composition of raft-enriched (raft) and raft-depleted PM (RDPM) of hepatocytes from trout *Oncorhynchus mykiss* acclimated to 5°C and 20°C. In the raft, warm acclimation was associated with a reduction in the proportion of phosphatidylcholine from 56% to 30% while phosphatidylserine and phosphatidylinositol each increased from 8% to approximately 20% of the total phospholipid. Additionally, there were significantly fewer unsaturated fatty acids in the raft lipids from warm-acclimated (61%) than from the cold-acclimated trout (68%). In contrast, there were no significant changes in phospholipid class or acyl chain unsaturation in the RDPM. These data suggest that changes in raft lipid composition, rather than the PM as a whole, are particularly important during thermal acclimation.

3.687 A primate virus generates transformed human cells by fusion

Duelli, D.M., Hearn, S., Myers, M.P. and Lazebnik, Y.
J. Cell Biol., ***171(3)***, *493-503 (2005)*

Amodel that explains both the origin and sporadic nature of cancer argues that cancer cells are a chance result of events that cause genomic and epigenetic variability. The prevailing view is that these events are

mutations that affect chromosome segregation or stability. However, genomic and epigenetic variability is also triggered by cell fusion, which is often caused by viruses. Yet, cells fused by viruses are considered harmless because they die. We provide evidence that a primate virus uses both viral and exosomal proteins involved in cell fusion to produce transformed proliferating human cells. Although normal cells indeed fail to proliferate after fusion, expression of an oncogene or a mutated tumor suppressor p53 in just one of the fusion partners is sufficient to produce heterogeneous progeny. We also show that this virus can produce viable oncogenically transformed cells by fusing cells that are otherwise destined to die. Therefore, we argue that viruses can contribute to carcinogenesis by fusing cells.

3.688 Plasma membrane and lysosomal localization of CB1 cannabinoid receptor are dependent on lipid rafts and regulated by anandamide in human breast cancer cells
Sarnataro, D. et al
FEBS Lett., ***579***, *6343-6349 (2005)*

In this report we show, by confocal analysis of indirect immunofluorescence, that the type-1 cannabinoid receptor (CB1R), which belongs to the family of G-protein-coupled receptors, is expressed on the plasma membrane in human breast cancer MDA-MB-231 cells. However, a substantial proportion of the receptor is present in lysosomes. We found that CB1R is associated with cholesterol- and sphyngolipid-enriched membrane domains (rafts). Cholesterol depletion by methyl-β-cyclodextrin (MCD) treatment strongly reduces the flotation of the protein on the raft-fractions (DRM) of sucrose density gradients suggesting that CB1 raft-association is cholesterol dependent. Interestingly binding of the agonist, anandamide (AEA) also impairs DRM-association of the receptor suggesting that the membrane distribution of the receptor is dependent on rafts and is possibly regulated by the agonist binding. Indeed MCD completely blocked the clustering of CB1R at the plasma membrane. On the contrary the lysosomal localization of CB1R was impaired by this treatment only after AEA binding.

3.689 Effect of cholesterol depletion on mitogenesis and survival: the role of caveolar and noncaveolar domains in insulin-like growth factor-mediated cellular function
Matthews, L.C., Taggart, M.J. and Westwood, M.
Endocrinol., ***146(12)***, *5463-5473 (2005)*

The type 1 IGF receptor (IGF-IR) is thought to localize to a subset of lipid rafts, known as caveolae, but the impact on IGF signaling remains controversial. We investigated this potential regulatory mechanism by assessing IGF function in caveolae-positive (3T3L1 and NWTb3) and -negative (HepG2) cells. Coimmunoprecipitation studies demonstrated that IGF-IR and insulin receptor substrate 1 associated with caveolin, a caveolar marker, in 3T3L1 and NWTb3 cells. Subcellular fractionation showed that methyl-cyclodextrin, which disrupts lipid rafts by sequestration of cholesterol, disrupted the colocalization of caveolin and the IGF-IR at the plasma membrane. Methyl-cyclodextrin did not alter IGF-I-induced 3T3L1 or NWTb3 proliferation but significantly impaired the ability of IGF-I to protect these cells from apoptosis. Immunoblotting revealed that methyl-cyclodextrin had no effect on IGF-I-induced activation of the IGF-IR or insulin receptor substrate 1 but increased and decreased the phosphorylation of MAPK and protein kinase B, respectively. In caveolae-negative HepG2 cells, the effect of methyl-cyclodextrin on IGF signaling and cellular function was similar to that observed in caveolae-positive 3T3L1 and NWTb3 cells. Furthermore, transfecting caveolin into HepG2 cells to give morphologically identifiable caveolae made no difference to IGF action, despite a demonstrable interaction between caveolin and the IGF-IR. This suggests that although IGF-IR localizes to caveolin-rich subcellular fractions and coimmunoprecipitates with caveolin, caveolae may not be obligatory for IGF signaling.

3.690 The ubiquitously expressed Csk adaptor protein Cbp is dispensable for embryogenesis and T-cell development and function
Dobenecker, M-W., Schmedt, C., Okada, M. And Tarakhovsky, A.
Mol. Cell. Biol., ***25(23)***, *10533-10542 (2005)*

Regulation of Src family kinase (SFK) activity is indispensable for a functional immune system and embryogenesis. The activity of SFKs is inhibited by the presence of the carboxy-terminal Src kinase (Csk) at the cell membrane. Thus, recruitment of cytosolic Csk to the membrane-associated SFKs is crucial for its regulatory function. Previous studies utilizing in vitro and transgenic models suggested that the Csk-binding protein (Cbp), also known as phosphoprotein associated with glycosphingolipid microdomains (PAG), is the membrane adaptor for Csk. However, loss-of-function genetic evidence to support this notion was lacking. Herein, we demonstrate that the targeted disruption of the *cbp* gene in mice has no effect on

embryogenesis, thymic development, or T-cell functions in vivo. Moreover, recruitment of Csk to the specialized membrane compartment of "lipid rafts" is not impaired by Cbp deficiency. Our results indicate that Cbp is dispensable for the recruitment of Csk to the membrane and that another Csk adaptor, yet to be discovered, compensates for the loss of Cbp.

3.691 Different effects of intracochlear sensory and neuronal injury stimulation on expression of synaptic N-methyl-D-aspartate receptors in the auditory cortex of rats in vivo
Wang, Z., Ruan, Q. and Wang, D.
*Acta Oto-Laryngologica, **125**, 1145-1151 (2005)*

Conclusions. The expression of synaptic N-methyl-D-aspartate (NMDA) receptors in the auditory cortex is dynamic and is bidirectionally regulated by auditory activity. Furthermore, the time course of changes in the level of NR2A protein differs after sensory and neuronal injury stimulation, which modulate different changes in synaptic plasticity. Objective. To examine the effects of different types of auditory activity on the expression of synaptic NMDA receptors (NMDARs) in the auditory cortex of rats. Material and methods. We prepared synaptosomes from the auditory cortices of postnatal Day 28 ototoxic-deafened Sprague–Dawley rats and postnatal Day 28 Sprague–Dawley rats subjected to noise trauma that were given various treatments and compared them to the synaptosomes of 1–6-week-old normal Sprague–Dawley rats. The expression of different NMDAR subunits in the synaptosomes was investigated by means of Western blotting. Results. Changes in NR1 and NR2B proteins were not significant during different types of auditory activity. The level of NR2A protein increased remarkably during postnatal development and as a result of electrical intracochlear stimulation, auditory deprivation and noise trauma. Seventy-two h after a 2-h period of sensory electrical intracochlear stimulation, the expression of NR2A protein returned to the level caused by auditory deprivation. Seventy-two h after a 3-h period of noise trauma, elevation of the level of NR2A protein was unchanged. We also confirmed that elevation of the level of synaptic NR2A protein was sensitive to protein synthesis inhibitor and NMDAR antagonist. However, transcription inhibitor had no effect on NR2A protein expression.

3.692 Defective insulin receptor activation and altered lipid rafts in Niemann-Pick type C diseases hepatocytes
Vainio, S. et al
*Biochem. J., **391**, 465-472 (2005)*

Niemann–Pick type C (NPC) disease is a neuro-visceral cholesterol storage disorder caused by mutations in the *NPC-1* or *NPC-2* gene. In the present paper, we studied IR (insulin receptor) activation and the plasma-membrane lipid assembly in primary hepatocytes from control and NPC1–/– mice. We have previously reported that, in hepatocytes, IR activation is dependent on cholesterol–sphingolipid rafts [Vainio, Heino, Mansson, Fredman, Kuismanen, Vaarala and Ikonen (2002) EMBO Rep. **3**, 95–100]. We found that, in NPC hepatocytes, IR levels were up-regulated and the receptor activation was compromised. Defective IR activation was reproduced in isolated NPC plasma-membrane preparations, which displayed an increased cholesterol content and saturation of major phospholipids. The NPC plasma membranes were less fluid than control membranes as indicated by increased DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence anisotropy values. Both in NPC hepatocytes and plasma-membrane fractions, the association of IR with low-density DRMs (detergent-resistant membranes) was increased. Moreover, the detergent resistance of both cholesterol and phosphatidylcholine were increased in NPC membranes. Finally, cholesterol removal inhibited IR activation in control membranes but restored IR activation in NPC membranes. Taken together, the results reveal a lipid imbalance in the NPC hepatocyte, which increases lipid ordering in the plasma membrane, alters the properties of lipid rafts and interferes with the function of a raft-associated plasma-membrane receptor. Such a mechanism may participate in the pathogenesis of NPC disease and contribute to insulin resistance in other disorders of lipid metabolism.

3.693 Malonyl-CoA decarboxylase is present in the cytosolic, mitochondrial and peroxisomal compartments of rat hepatocytes
Joly, E. et al

FEBS Lett., ***579(29)***, *6581-6586 (2005)*

A role for cytosolic malonyl-CoA decarboxylase (MCD) as a regulator of fatty acid oxidation has been postulated. However, there is no direct evidence that MCD is present in the cytosol. To address this issue, we performed cell fractionation and electron microscopic colloidal gold studies of rat liver to determine the location and activity of MCD. By both methods, substantial amounts of MCD protein and activity were found in the cytosol, mitochondria and peroxisomes, the latter with the highest specific activity. MCD species with different electrophoretic mobility were observed in the three fractions. The data demonstrate that active MCD is present in the cytosol, mitochondria and peroxisomes of rat liver, consistent with the view that MCD participates in the regulation of cytosolic malonyl-CoA levels and of hepatic fatty acid oxidation.

3.694 **Defects in structural integrity of ergosterol and the Cdc50p-Drs2p putative phospholipid translocase cause accumulation of endocytic membranes, onto which actin parches are assembled in yeast**
Kishimoto, T., Yamamoto, T. and Tanaka, K.
Mol. Biol. Cell, ***16***, *5592-5609 (2005)*

Specific changes in membrane lipid composition are implicated in actin cytoskeletal organization, vesicle formation, and control of cell polarity. Cdc50p, a membrane protein in the endosomal/*trans*-Golgi network compartments, is a noncatalytic subunit of Drs2p, which is implicated in translocation of phospholipids across lipid bilayers. We found that the *cdc50*Δ mutation is synthetically lethal with mutations affecting the late steps of ergosterol synthesis (*erg2* to *erg6*). Defects in cell polarity and actin organization were observed in the *cdc50*Δ *erg3*Δ mutant. In particular, actin patches, which are normally found at cortical sites, were assembled intracellularly along with their assembly factors, including Las17p, Abp1p, and Sla2p. The exocytic SNARE Snc1p, which is recycled by an endocytic route, was also intracellularly accumulated, and inhibition of endocytic internalization suppressed the cytoplasmic accumulation of both Las17p and Snc1p. Simultaneous loss of both phospholipid asymmetry and sterol structural integrity could lead to accumulation of endocytic intermediates capable of initiating assembly of actin patches in the cytoplasm.

3.695 **A genome-wide visual screen reveals a role for sphingolipids and ergosterol in cell surface delivery in yeast**
Proszynski, T.J. et al
PNAS, ***102(50)***, *17981-17986 (2005)*

Recently synthesized proteins are sorted at the trans-Golgi network into specialized routes for exocytosis. Surprisingly little is known about the underlying molecular machinery. Here, we present a visual screen to search for proteins involved in cargo sorting and vesicle formation. We expressed a GFP-tagged plasma membrane protein in the yeast deletion library and identified mutants with altered marker localization. This screen revealed a requirement of several enzymes regulating the synthesis of sphingolipids and ergosterol in the correct and efficient delivery of the marker protein to the cell surface. Additionally, we identified mutants regulating the actin cytoskeleton (Rvs161p and Vrp1p), known membrane traffic regulators (Kes1p and Chs5p), and several unknown genes. This visual screening method can now be used for different cargo proteins to search in a genome-wide fashion for machinery involved in post-Golgi sorting.

3.696 **Expression and activity of NOX5 in the circulating malignant B cells of hairy cell leukemia**
Kamiguti, A.S. et al
J. Immunol., ***175***, *8424-8430 (2005)*

Hairy cells (HCs) are mature malignant B cells that contain a number of constitutively active signaling molecules including GTP-bound Rac1, protein kinase C, and Src family kinases. Because Rac1 is a component of the reactive oxidant species (ROS)-generating NADPH oxidase system, we investigated the role of this GTPase in ROS production in HCs. In this study, we show that ROS production in HCs involves a flavin-containing oxidase dependent on Ca^{2+}, but not on GTP-Rac1 or protein kinase C. This suggests the involvement of the nonphagocytic NADPH oxidase NOX5, an enzyme found in lymphoid tissues, but not in circulating lymphocytes. By using RT-PCR and Southern and Western blotting and by measuring superoxide anion production in membrane fractions in the absence of cytosolic components, we

demonstrate for the first time that HCs (but not circulating normal B cells or some other lymphoid cell types) express NOX5. We also demonstrate that inhibition of NADPH oxidase in HCs results in a selective increase in the activity of Src homology region 2 domain-containing phosphatase 1 (SHP-1). Furthermore, SHP-1 in HCs coimmunoprecipitates with tyrosine phosphorylated CD22 and localizes in the same cellular compartment as NOX5. This allows the inactivation of SHP-1 by NOX5-generated ROS and contributes to the maintenance of the constitutive activation of HCs.

3.697 Solute traffic across mammalian peroxisomal membrane – single-channel conductance monitoring reveals pore-forming activities in peroxisomes
Antonenkov, V.D., Rokka, A., Sormunen, R.T., Benz, R. And Hiltunen, J.K.
Cell. Mol. Life Sci., ***62***, *2886-2895 (2005)*

Mouse liver peroxisomes were isolated by centrifugation in a self-generated Percoll gradient followed by an Optiprep density gradient centrifugation. Peroxisomes contributed 90–96% of the total protein content in the fraction, as confirmed by marker enzyme assays, protein pattern in SDS-PAGE, immunoblotting, and electron microscopy. Solubilized peroxisomal membrane proteins were reconstituted into a planar lipid bilayer. A single-channel conductance monitoring of the reconstituted lipid bilayer revealed the presence of two pore-forming components with a conductance in 1 M KCl of 1.3 nS and 2.5 nS. Control experiments with fractions enriched in mitochondria, lysosomes, and fragments of endoplasmic reticulum showed that the peroxisomal channel-forming activities were not due to admixture of isolated peroxisomes with other cellular organelles. The peroxisomal channels were well preserved in membrane preparations but became unstable after solubilization from the membranes by detergent.

3.698 The compartmentalization of prolactin signaling in the mouse mammary gland
Bolander Jr., F.F.
Mol. Cell. Endocrinol., ***245(1-2)***, *105-110 (2005)*

In mammary epithelial cells, prolactin (PRL) activates at least two signaling pathways: Jak/Stat and nitric oxide (NO). The former induces differentiation as measured by α-lactalbumin accumulation, while experiments with sodium nitroprusside (SNP) show that NO inhibits differentiation. In order to resolve this apparent contradiction, the kinetics, inducibility, and cellular localization of NO production and sensitivity in mammary cells were examined. First, mammary cells remained responsive to PRL throughout the incubation with respect to NO production. Second, although desensitization occurred with continuous PRL exposure, recovery began as quickly as 30 min after PRL withdrawal. Since PRL is secreted in pulses in vivo, complete desensitization was not a likely explanation for the cells' escape from NO inhibition. Finally, the cellular site of transduction was examined using the caveolar disrupting agent, methyl-β-cyclodextrin (MBCD). MBCD inhibited the accumulation of PRL-induced NO but not α-lactalbumin. This finding was confirmed by membrane fractionation studies where the PRL-induced NO production occurred primarily in caveolae and PRL-stimulated tyrosine phosphorylation of Stat5, which transcribes the α-lactalbumin gene, occurred predominantly in noncaveolar membranes. Finally, endogenous elevations of NO by arginine did not inhibit differentiation. As such, the inhibition seen with SNP appeared to be an artifact of the ubiquitous generation of NO from SNP. Physiologically, PRL induces NO only in caveolae and this restricted distribution does not interfere with differentiation.

3.699 Oestrogen-mediated tyrosine phosphorylation of caveolin-1 and its effect on the oestrogen receptor localization: an in vivo study
Kiss, A.L. et al
Mol. Cell. Endocrinol., ***245(1-2)***, *128-137 (2005)*

Recently, it has been shown that 17β estradiol (E2) induces a rapid and transient activation of the Src ERK phosphorylation cascade: a clear indication that the α oestrogen receptor (ERα) is able to associate with the plasma membrane. Increasing evidence suggests that caveolae, which are caveolin-1 containing, highly hydrophobic membrane domains, play an important role in E2 induced signal transduction. Caveolae can accumulate signalling molecules preferentially; thus, they may have a regulatory role in signalling processes. Results from previous experiments have shown that E2 treatment decreased the number of surface connected caveolae significantly in uterine smooth muscle cells and also downregulated the expression of caveolin-1. In addition to providing further evidence that ERα interacts with caveolin/caveolae in uterine smooth muscle cells, this study also shows that the interaction between caveolin-1 and ERα is actually facilitated by E2. One of the signal transduction components found to accumulate in caveolae is Src kinase in an amount that increases simultaneously with increases in the

amount of ERα. Upon E2 treatment, Src kinase is tyrosine phosphorylated, which, in turn, stimulates Src kinase to phosphorylate caveolin-1. Phosphorylation of caveolin-1 can drive caveolae to pinch off from the plasma membrane, thereby decreasing the amount of plasma membrane-associated caveolin-1. This loss of caveolin/caveolae activates the signal cascade that triggers cell proliferation.

3.700 Sorghum Genome Sequencing by Methylation Filtration
Bedell, J.A. et al
*PloS Biol., **3(1)**, 103-115 (2005)*

Sorghum bicolor is a close relative of maize and is a staple crop in Africa and much of the developing world because of its superior tolerance of arid growth conditions. We have generated sequence from the hypomethylated portion of the sorghum genome by applying methylation filtration (MF) technology. The evidence suggests that 96% of the genes have been sequence tagged, with an average coverage of 65% across their length. Remarkably, this level of gene discovery was accomplished after generating a raw coverage of less than 300 megabases of the 735-megabase genome. MF preferentially captures exons and introns, promoters, microRNAs, and simple sequence repeats, and minimizes interspersed repeats, thus providing a robust view of the functional parts of the genome. The sorghum MF sequence set is beneficial to research on sorghum and is also a powerful resource for comparative genomics among the grasses and across the entire plant kingdom. Thousands of hypothetical gene predictions in rice and *Arabidopsis* are supported by the sorghum dataset, and genomic similarities highlight evolutionarily conserved regions that will lead to a better understanding of rice and *Arabidopsis*.

3.701 T cell receptor-induced lipid raft recruitment of the IκB kinase complex is necessary and sufficient for NF-κB activation occurring in the cytosol
Sebald, A., Mattioli, I. and Schmitz, M.L.
*Eur. J. Immunol., **35(1)**, 318-325 (2005)*

TCR-induced NF-κB activation is necessary for the innate immune response and involves induced lipid raft recruitment of the IκB kinase (IKK) complex. In this study, we systematically investigated lipid raft recruitment of members of the NF-κB activation pathway in human T cells. All upstream components leading to IKK activation were found constitutively or inducibly in lipid rafts, while the NF-κB/IκB complex and phosphorylated forms of IKKα/β, IκBα and p65 are exclusively found in the cytosolic fraction. Disruption of raft organization precluded NF-κB activation induced by T cell costimulation, but IL-1-triggered NF-κB activation remained unaffected. Targeting of the IKK complex to lipid rafts caused constitutive IKK activation and NF-κB DNA binding, which was further triggered upon T cell costimulation. Various experimental approaches revealed that costimulation-induced IKKα/β activation loop phosphorylation is independent from IKKβ-mediated transautophosphorylation, but rather involves phosphorylation by the IKK-interacting protein NIK and its upstream activator COT.

3.702 *Leishmania donovani* lipophosphoglycan disrupts phagosome microdomains in J774 macrophages
Dermine. J-F., Goyette, G., Houde, M., Turco, S.J. and Desjardins, M.
*Cell. Microbiol., **7(9)**, 1263-1270 (2005)*

Clearance of pathogens by phagocytosis and their killing in phagolysosomes is a key aspect of our innate ability to fight infectious agents. Leishmania parasites have evolved ways to survive and replicate in macrophages by inhibiting phagosome maturation and avoiding the harsh environment of phagolysosomes. We describe here that during this process Leishmania donovani uses a novel strategy involving its surface lipophosphoglycan (LPG), a virulence factor impeding many host functions, to prevent the formation or disrupt lipid microdomains on the phagosome membrane. LPG acts locally on the membrane and requires its repetitive carbohydrate moieties to alter the organization of microdomains. Targeting and disruption of functional foci, where proteins involved in key aspects of phagolysosome biogenesis assemble, is likely to confer a survival advantage to the parasite.

3.703 Intragranular Vesiculotubular Compartments are Involved in Piecemeal Degranulation by Activated Human Eosinophils
Melo, R.C.N., Perez, S.A.C., Spencer, L.A., Dvorak, A.M. and Weller, P.F.
*Traffic, **6(10)**, 866-879 (2005)*

Eosinophils, leukocytes involved in allergic, inflammatory and immunoregulatory responses, have a distinct capacity to rapidly secrete preformed granule-stored proteins through piecemeal degranulation (PMD), a secretion process based on vesicular transport of proteins from within granules for extracellular release. Eosinophil-specific granules contain cytokines and cationic proteins, such as major basic protein (MBP). We evaluated structural mechanisms responsible for mobilizing proteins from within eosinophil granules. Human eosinophils stimulated for 30–60 min with eotaxin, regulated on activation, normal, T-cell expressed and secreted (RANTES) or platelet activating factor exhibited ultrastructural features of PMD (e.g. losses of granule contents) and extensive vesiculotubular networks within emptying granules. Brefeldin A inhibited granule emptying and collapsed intragranular vesiculotubular networks. By immunonanogold ultrastructural labelings, CD63, a tetraspanin membrane protein, was localized within granules and on vesicles outside of granules, and mobilization of MBP into vesicles within and extending from granules was demonstrated. Electron tomography with three dimension reconstructions revealed granule internal membranes to constitute an elaborate tubular network able to sequester and relocate granule products upon stimulation. We provide new insights into PMD and identify eosinophil specific granules as organelles whose internal tubulovesicular networks are important for the capacity of eosinophils to secrete, by vesicular transport, their content of preformed and granule-stored cytokines and cationic proteins.

3.704 Peroxisomal proteomics, a new tool for risk assessment of peroxisome proliferating pollutants in the marine environment

Mi, J., Orbea, A., Syme, N., Ahmed, M., Cajaraville, M.P. and Cristobal, S.
*Proteomics, **5(15)**, 3954-2965 (2005)*

In an attempt to improve the detection of peroxisome proliferation as a biomarker in environmental pollution assessment, we have applied a novel approach based on peroxisomal proteomics. Peroxisomal proteins from digestive glands of mussels *Mytilus galloprovincialis* were analyzed using 2-DE and MS. We have generated a reference 2-DE map from samples obtained in a well-studied reference area and compared this with peroxisomal proteomes from other sequenced genomes. In addition, by comparing 2-DE maps from control samples with samples obtained in a polluted area, we have characterized the peroxisome proliferation expression pattern associated with exposure to a polluted environment. Over 100 spots were reproducibly resolved per 2-DE map; 55 differentially expressed spots were quantitatively detected and analyzed, and 14 of these showed an increase in protein expression of more than fourfold. Epoxide hydrolase, peroxisomal antioxidant enzyme, and sarcosine oxidase (SOX) have been identified by ESI MS/MS, and acyl-CoA oxidase, multifunctional protein, and Cu,Zn-superoxide dismutase were immunolocalized by Western blotting. Our results indicate that a peroxisomal protein pattern associated to marine pollutant exposure can be generated, and this approach may have a greater potential as biomarker than traditional, single-protein markers.

3.705 Altered regulation of EGF receptor signaling following a partial hepatectomy

Skarpen, E., Oksvold, M.P., Grøsvik, H., Widnes, C. and Huitfeldt, H.S.
*J. Cell. Physiol., **202(3)**, 707-716 (2005)*

We have studied epidermal growth factor receptor (EGFR) phosphorylation and localization in the pre-replicative phase of liver regeneration induced by a 70% partial hepatectomy (PH), and how a PH affects EGFR activation and trafficking. When Western blotting was performed on livers after PH with antibodies raised against activated forms of EGFR autophosphorylation sites, no marked increase in EGFR tyrosine phosphorylation was observed. However, events associated with attenuation of EGFR signals were observed. Two hours after PH, we found increased EGFR ubiquitination and internalization, followed by receptor downregulation. Furthermore, EGFR phosphorylation following an injection of EGF was reduced after PH. This reduction correlated with an increased activation of PKC and a distinct augmentation in the phosphorylation of the PKC-regulated T654-site of EGFR. When primary cultured hepatocytes were treated with tetradecanoylphorbol acetate (TPA) to induce T654-phosphorylation of EGFR, we found colocalization of a fraction of EGFR with EEA1, downregulation of EGF-mediated EGFR autophosphorylation, altered ligand-induced intracellular sorting of EGFR, and increased mitogenic signaling through the EGFR-Ras-Raf-ERK pathway. Further, we found that both TPA and a PH enhanced EGF-induced proliferation of hepatocytes. In conclusion, our results suggest that hepatocyte priming involves modulation of EGFR that enhances its ability to mediate growth factor responses without an increase in its receptor tyrosine kinase-activity. This may be a pre-replicative competence event that increases growth factor effects during G1 progression.

3.706 Characterization of myosin-II binding to Golgi stacks in vitro
Fath, K.R.
Cell Motil. Cytoskeleton, ***60(4)****, 222-235 (2005)*

In addition to important roles near the actin-rich cell cortex, ample evidence indicates that multiple myosins are also involved in membrane movements in the endomembrane system. Nonmuscle myosin-II has been shown to have roles in anterograde and retrograde trafficking at the Golgi. Myosin-II is present on Golgi stacks isolated from intestinal epithelial cells and has been localized to the Golgi in several polarized and unpolarized cell lines. An understanding of roles of myosin-II in Golgi physiology will be facilitated by understanding the molecular arrangement of myosin-II at the Golgi. Salt-washing removes endogenous myosin-II from isolated Golgi and purified brush border myosin-II can bind in vitro. Brush border myosin-II binds to a tightly bound Golgi peripheral membrane protein with a $K_{1/2}$ of 75 nM and binding is saturated at 0.7 pmol myosin/μg Golgi. Binding studies using papain cleavage fragments of brush border myosin-II show that the 120-kDa rod domain, but not the head domain, of myosin heavy chain can bind directly to Golgi stacks. The 120-kDa domain does not bind to Golgi membranes when phosphorylated in vitro with casein kinase-II. These results suggest that phosphorylation in the rod domain may regulate the binding and/or release of myosin-II from the Golgi. These data support a model in which myosin-II is tethered to the Golgi membrane by its tail and actin filaments by its head. Thus, translocation along actin filaments may extend Golgi membrane tubules and/or vesicles away from the Golgi complex.

3.707 The unconventional myosin-VIIa associates with lysosomes
Soni, L.E., Warren, C.M., Bucci, C., Orten, D.J. and Hasson, T.
Cell Motil. Cytoskeleton, ***62(1)****, 13-26 (2005)*

Mutations in the myosin-VIIa (*MYO7a*) gene cause human Usher disease, characterized by hearing impairment and progressive retinal degeneration. In the retina, myosin-VIIa is highly expressed in the retinal pigment epithelium, where it plays a role in the positioning of melanosomes and other digestion organelles. Using a human cultured retinal pigmented epithelia cell line, ARPE-19, as a model system, we have found that a population of myosin-VIIa is associated with cathepsin D- and Rab7-positive lysosomes. Association of myosin-VIIa with lysosomes was Rab7 independent, as dominant negative and dominant active versions of Rab7 did not disrupt myosin-VIIa recruitment to lysosomes. Association of myosin-VIIa with lysosomes was also independent of the actin and microtubule cytoskeleton. Myosin-VIIa copurified with lysosomes on density gradients, and fractionation and extraction experiments suggested that it was tightly associated with the lysosome surface. These studies suggest that myosin-VIIa is a lysosome motor.

3.708 Release of the type I secreted α-haemolysin via outer membrane vesicles from *Escherichia coli*
Balsalobre, C. et al
Mol. Microbiol., ***59(1)****, 99-112 (2006)*

The α-haemolysin is an important virulence factor commonly expressed by extraintestinal pathogenic *Escherichia coli*. The secretion of the α-haemolysin is mediated by the type I secretion system and the toxin reaches the extracellular space without the formation of periplasmic intermediates presumably in a soluble form. Surprisingly, we found that a fraction of this type I secreted protein is located within outer membrane vesicles (OMVs) that are released by the bacteria. The α-haemolysin appeared very tightly associated with the OMVs as judged by dissociation assays and proteinase susceptibility tests. The α-haemolysin in OMVs was cytotoxically active and caused lysis of red blood cells. The OMVs containing the α-haemolysin were distinct from the OMVs not containing α-haemolysin, showing a lower density. Furthermore, they differed in protein composition and one component of the type I secretion system, the TolC protein, was found in the lower density vesicles. Studies of natural isolates of *E. coli* demonstrated that the localization of α-haemolysin in OMVs is a common feature among haemolytic strains. We propose an alternative pathway for the transport of the type I secreted α-haemolysin from the bacteria to the host cells during bacterial infections.

3.709 Nox2 and Rac1 regulate H_2O_2-dependent recruitment of TRAFg to endosomal interleukin-1 receptor complexes
Li, Q. et al
Mol. Cell. Biol., ***26(1)****, 140-154 (2006)*

Reactive oxygen species (ROS) generated by NADPH oxidases (Nox) have been implicated in the regulation of signal transduction. However, the cellular mechanisms that link Nox activation with plasma membrane receptor signaling remain poorly defined. We have found that Nox2-derived ROS influence the formation of an active interleukin-1 (IL-1) receptor complex in the endosomal compartment by directing the H_2O_2-dependent binding of TRAF6 to the IL-1R1/MyD88 complex. Clearance of both superoxide and H_2O_2 from within the endosomal compartment significantly abrogated IL-1ß-dependent IKK and NF-κB activation. MyD88-dependent endocytosis of IL-1R1 following IL-1ß binding was required for the redox-dependent formation of an active endosomal receptor complex competent for IKK and NF-κB activation. Small interfering RNAs to either MyD88 or Rac1 inhibited IL-1ß induction of endosomal superoxide and NF-κB activation. However, MyD88 and Rac1 appear to be recruited independently to IL-1R1 following ligand stimulation. In this context, MyD88 binding was required for inducing endocytosis of IL-1R1 following ligand binding, while Rac1 facilitated the recruitment of Nox2 into the endosomal compartment and subsequent redox-dependent recruitment of TRAF6 to the MyD88/IL-1R1 complex. The identification of Nox-active endosomes helps explain how subcellular compartmentalization of redox signals can be used to direct receptor activation from the plasma membrane.

3.710 Lipid raft clustering and redox signaling platform formation in coronary arterial endothelial cells
Zhung, A.Y., Yi, F., Zhang, G., Gulbins, E. and Li, P-L.
*Hypertension, **47**, 74-80 (2006)*

Recent studies have indicated that lipid rafts (LRs) in the cell membrane are clustered in response to different stimuli to form signaling platforms for transmembrane transduction. It remains unknown whether this LR clustering participates in redox signaling in endothelial cells. The present study tested a hypothesis that clustering of LRs on the membrane of coronary endothelial cells produces aggregation and activation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, thereby forming a redox signaling platform. By confocal microscopic analysis of agonist-stimulated rafts patch formation, we found that several death receptor ligands or apoptotic factors, including tumor necrosis factor [alpha], Fas ligand, or endostatin, stimulated the clustering and trafficking of individual LRs on the plasma membrane of coronary endothelial cells. Interestingly, double labeling of a membrane-bound NADPH oxidase subunit, gp91phox, and LRs showed that gp91phox colocalized within the LR patches when endothelial cells were stimulated by Fas ligand. In isolated LR fractions from Fas-stimulated endothelial cells, gp91phox, p47phox (a crucial cytosolic regulatory subunit of NADPH oxidase), and Rac GTPase were markedly increased and blocked by nystatin, a compound that disrupts LRs. These clustered LRs contained high NADPH oxidase activity, which increased in response to Fas stimulation. Functionally, Fas ligand-induced inhibition of endothelium-dependent vasorelaxation was reduced if LRs were disrupted or NADPH oxidase was inhibited. These results suggest that LR clustering occurs in coronary endothelial cells. The formation of redox signaling platforms on the cell membrane mediates transmembrane signaling of death receptors, resulting in endothelial dysfunction.

3.711 Significance of sterol structural specificity
Vainio, S. et al
*J. Biol. Chem., **281(1)**, 348-355 (2006)*

Desmosterol is an immediate precursor of cholesterol in the Bloch pathway of sterol synthesis and an abundant membrane lipid in specific cell types. The significance of the difference between the two sterols, an additional double bond at position C24 in the tail of desmosterol, is not known. Here, we provide evidence that the biophysical and functional characteristics of the two sterols differ and that this is because the double bond at C24 significantly weakens the sterol ordering potential. In model membranes, desmosterol was significantly weaker than cholesterol in promoting the formation or stability of ordered domains, and in mammalian cell membranes, desmosterol associated less avidly than cholesterol with detergent-resistant membranes. Atomic scale molecular dynamics simulations showed that the double bond gives rise to additional stress in the tail, creating a rigid structure between C24 and C27 and favoring tilting of desmosterol distinct from cholesterol. Functional effects of desmosterol in cell membranes were assessed upon acutely exchanging ~70% of cholesterol to desmosterol. This led to impaired raft-dependent signaling via the insulin receptor, whereas non-raft-dependent protein secretion was not affected. We suggest that the choice of cholesterol synthesis route may provide a physiological mechanism to modulate raft-dependent functions in cells.

3.712 Detection of a raft-located estrogen receptor-like protein distinct from ERα
Heberden, C. et al

*Int. J. Biochem. Cell Biol., **38**, 376-391 (2006)*

17β-Estradiol (17β-E2) elicits at the cell membrane rapid actions that remain insensitive to the inhibitory effect of ICI 182,780, a pure estrogen antagonist, and therefore cannot be attributed to the classic nuclear receptors. We addressed the question of the identity of the protein involved in these rapid actions. We first examined the responses of several cell lines for intracellular calcium mobilization, an effect not inhibited by ICI 182,780, tamoxifen and raloxifen. We then demonstrated the presence of binding sites in the membranes, by incubating them with antibodies directed against different domains of ERα, and by flow cytometry analysis. The membrane proteins were eluted by affinity chromatography using E2 conjugated to bovine serum albumin as a ligand. Western blots of the elution fractions using an antibody directed against the ligand binding site of ERα showed the existence of a protein of ~50 kDa. The protein was concentrated in the lipid rafts, together with another heavier form of ~66 kDa. The 50 kDa protein was immunoprecipitable, and co-immunoprecipitation experiments showed that it was associated with the $G\beta_{1-4}$ protein, but not with caveolin-1. The protein was expressed in ERα-null cells, like HO-23 and Cos-7 cells. Therefore, in the lipid rafts, there exists a protein, similar to, but molecularly distinct from ERα..

3.713 Identification of a switch in neurotrophin signaling by selective tyrosine phosphorylation
Arevalo, J., Pereira, D.B., Yano, H., Teng, K.K. and Chao, M.V.
*J. Biol. Chem., **281(2)**, 1001-1007 (2006)*

Neurotrophins, such as nerve growth factor and brain-derived neurotrophic factor, activate Trk receptor tyrosine kinases through receptor dimerization at the cell surface followed by autophosphorylation and recruitment of intracellular signaling molecules. The intracellular pathways used by neurotrophins share many common protein substrates that are used by other receptor tyrosine kinases (RTK), such as Shc, Grb2, FRS2, and phospholipase C-γ. Here we describe a novel RTK mechanism that involves a 220-kilodalton membrane tetraspanning protein, ARMS/Kidins220, which is rapidly tyrosine phosphorylated in primary neurons after neurotrophin treatment. ARMS/Kidins220 undergoes multiple tyrosine phosphorylation events and also serine phosphorylation by protein kinase D. We have identified a single tyrosine (Tyr^{1096}) phosphorylation event in ARMS/Kidins220 that plays a critical role in neurotrophin signaling. A reassembled complex of ARMS/Kidins220 and CrkL, an upstream component of the C3G-Rap1-MAP kinase cascade, is SH3-dependent. However, Tyr^{1096} phosphorylation enables ARMS/Kidins220 to recruit CrkL through its SH2 domain, thereby freeing the CrkL SH3 domain to engage C3G for MAP kinase activation in a neurotrophin dependent manner. Accordingly, mutation of Tyr^{1096} abolished CrkL interaction and sustained MAPK kinase activity, a response that is not normally observed in other RTKs. Therefore, Trk receptor signaling involves an inducible switch mechanism through an unconventional substrate that distinguishes neurotrophin action from other growth factor receptors.

3.714 From assembly to virus particle budding: pertince of the detergent resisitant membranes
Gosselin-Grenet, A-S., Mottet-Osman, G. and Roux, L.
*Virology, **344(2)**, 296-303 (2006)*

Detergent resistant membranes (DRMs) are the site of assembly for a variety of viruses. Here, we make use of Sendai virus mutant proteins that are not packaged into virus particles to determine the involvement of this assembly for the virus particle production. We found that, in the context of an infection, (1) all the Sendai virus proteins associated in part with DRMs, (2) mutant HN and M proteins not packaged into virus particles were similarly part of this association, (3) after M protein suppression resulting in a significant reduction of virus production, the floatation profile of the other viral proteins was not altered and finally (4) cellular cholesterol depletion did not decrease the virus particle production, although it somehow reduced their virus infectivity. These results led us to conclude that the assembly complex found in DRM fractions does not constitute a direct precursor of virus particle budding.

3.715 Roles of ZO-1, accludin, and actin in axidant-induced barrier disruption
Musch, M.W., Walsh-Reitz, M.W. and Chang, E.B.
*Am. J. Physiol., **290**, G222-G231 (2006)*

Oxidants such as monochloramine (NH_2Cl) decrease epithelial barrier function by disrupting perijunctional actin and possibly affecting the distribution of tight junctional proteins. These effects can, in theory, disturb cell polarization and affect critical membrane proteins by compromising molecular fence function of the tight junctions. To examine these possibilities, we investigated the actions of NH_2Cl on the distribution, function, and integrity of barrier-associated membrane, cytoskeletal, and adaptor proteins in human colonic

Caco-2 epithelial monolayers. NH_2Cl causes a time-dependent decrease in both detergent-insoluble and -soluble zonula occludens (ZO)-1 abundance, more rapidly in the former. Decreases in occludin levels in the detergent-insoluble fraction were observed soon after the fall of ZO-1 levels. The actin depolymerizer cytochalasin D resulted in a decreased transepithelial resistance (TER) more quickly than NH_2Cl but caused a more modest and slower reduction in ZO-1 levels and in occludin redistribution. No changes in the cellular distribution of claudin-1, claudin-5, or ZO-2 were observed after NH_2Cl. However, in subsequent studies, the immunofluorescent cellular staining pattern of all these proteins was altered by NH_2Cl. The actin-stabilizing agent phalloidin did not prevent NH_2Cl-induced decreases in TER or increases of apical to basolateral flux of the paracellular permeability marker mannitol. However, it partially blocked changes in ZO-1 and occludin distribution. Tight junctional fence function was also compromised by NH_2Cl, observed as a redistribution of the α-subunit of basolateral Na^+-K^+-ATPase to the apical membrane, an effect not found with the apical membrane protein Na^+/H^+ exchanger isoform 3. In conclusion, oxidants not only disrupt perijunctional actin but also cause redistribution of tight junctional proteins, resulting in compromised intestinal epithelial barrier and fence function. These effects are likely to contribute to the development of malabsorption and dysfunction associated with mucosal inflammation of the digestive tract.

3.716 Focal adhesion kinase is critical for entry of Kaposi's sarcoma-associated herpesvirus into target cells

Krisham, H.H., Sharma-Walia, N., Streblow, D.N., Naranatt, P.P. and Chandran, B.
J. Virol., ***80(3)***, *1167-1180 (2006)*

Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) interacts with cell surface α3ß1 integrin early during in vitro infection of human endothelial cells and fibroblasts and activates the focal adhesion kinase (FAK) that is immediately downstream in the outside-in signaling pathway by integrins, leading to the activation of several downstream signaling molecules. In this study, using real-time DNA and reverse transcription-PCR assays to measure total internalized viral DNA, viral DNA associated with infected nuclei, and viral gene expression, we examined the stage of infection at which FAK plays the most significant role. Early during KSHV infection, FAK was phosphorylated in FAK-positive Du17 mouse embryonic fibroblasts. The absence of FAK in Du3 ($FAK^{-/-}$) cells resulted in about 70% reduction in the internalization of viral DNA, suggesting that FAK plays a role in KSHV entry. Expression of FAK in Du3 ($FAK^{-/-}$) cells via an adenovirus vector augmented the internalization of viral DNA. Expression of the FAK dominant-negative mutant FAK-related nonkinase (FRNK) in Du17 cells significantly reduced the entry of virus. Virus entry in Du3 cells, albeit in reduced quantity, delivery of viral DNA to the infected cell nuclei, and expression of KSHV genes suggested that in the absence of FAK, another molecule(s) may be partially compensating for FAK function. Infection of Du3 cells induced the phosphorylation of the FAK-related proline-rich tyrosine kinase (Pyk2) molecule, which has been shown to complement some of the functions of FAK. Expression of an autophosphorylation site mutant of Pyk2 in which Y402 is mutated to F (F402 Pyk2) reduced viral entry in Du3 cells, suggesting that Pyk2 facilitates viral entry moderately in the absence of FAK. These results suggest a critical role for KSHV infection-induced FAK in the internalization of viral DNA into target cells.

3.717 Quality control of a mutant plasma membrane ATPase: ubiquitylation prevents cell-surface stability

Liu, Y. and Chang, A.
J. Cell Sci., ***119***, *360-369 (2006)*

The plasma membrane ATPase, Pma1, has remarkable longevity at the cell surface. In contrast to the wild-type protein, the temperature-sensitive mutant Pma1-10 is misfolded and undergoes rapid removal from the cell surface for vacuolar degradation. At the restrictive temperature, Pma1-10 becomes ubiquitylated before or upon arrival at the plasma membrane. Internalization from the plasma membrane and vacuolar degradation of Pma1-10 is dependent on the ubiquitin-interacting motif (UIM) of the epsin Ent1, suggesting recognition of ubiquitylated substrate by the endocytic machinery. Surprisingly, ubiquitylation of Pma1-10 is reversed when its internalization is blocked in an *end3* mutant. Under these conditions, Pma1-10 acquires association with detergent-insoluble, glycolipid-enriched complexes (DIGs) which has been suggested to promote stability of wild-type Pma1. Ubiquitylation does not cause DIG exclusion because a Pma1-Ub fusion protein is not significantly excluded from DIGs. We suggest that ubiquitylation of Pma1-10 represents a component of a quality control mechanism that targets the misfolded protein for removal from the plasma membrane. Rapid internalization of Pma1-10 caused by its ubiquitylation may preempt establishment of stabilizing interactions.

3.718 Protein kinase Cε interacts with cytochrome c oxidase subunit IV and enhances cytochrome c oxidase activity in neonatal cardiac myocyte preconditioning
Ogbi, M. and Johnson, J.A.
Biochem. J., ***393(1)***, *191-199 (2006)*

We have previously identified a phorbol ester-induced PKCε (protein kinase Cε) interaction with the (~18 kDa) COIV [CO (cytochrome *c* oxidase) subunit IV] in NCMs (neonatal cardiac myocytes). Since PKCε has been implicated as a key mediator of cardiac PC (preconditioning), we examined whether hypoxic PC could induce PKCε–COIV interactions. Similar to our recent study with phorbol esters [Ogbi, Chew, Pohl, Stuchlik, Ogbi and Johnson (2004) Biochem. J. **382**, 923–932], we observed a time-dependent increase in the *in vitro* phosphorylation of an approx. 18 kDa protein in particulate cell fractions isolated from NCMs subjected to 1–60 min of hypoxia. Introduction of a PKCε-selective translocation inhibitor into cells attenuated this *in vitro* phosphorylation. Furthermore, when mitochondria isolated from NCMs exposed to 30 min of hypoxia were subjected to immunoprecipitation analyses using PKCε-selective antisera, we observed an 11.1-fold increase in PKCε–COIV co-precipitation. In addition, we observed up to 4-fold increases in CO activity after brief NCM hypoxia exposures that were also attenuated by introducing a PKCε-selective translocation inhibitor into the cells. Finally, in Western-blot analyses, we observed a >2-fold PC-induced protection of COIV levels after 9 h index hypoxia. Our studies suggest that a PKCε–COIV interaction and an enhancement of CO activity occur in NCM hypoxic PC. We therefore propose novel mechanisms of PKCε-mediated PC involving enhanced energetics, decreased mitochondrial reactive oxygen species production and the preservation of COIV levels.

3.719 CD16b associates with high-density, detergent-resistant membranes in human neutrophils
Fernandes, M.J.G. et al
Biochem. J., ***393(2)***, *351-359 (2006)*

CD16b is unique in that it is the only Fc receptor linked to the plasma membrane by a GPI (glycosylphosphatidylinositol) anchor. GPI-anchored proteins often preferentially localize to DRMs (detergent-resistant membranes) that are rich in sphingolipids and cholesterol and play an important role in signal transduction. Even though the responses to CD16b engagement have been intensively investigated, the importance of DRM integrity for CD16b signalling has not been characterized in human neutrophils. We provide direct evidence that CD16b constitutively partitions with both low- and high-density DRMs. Moreover, upon CD16b engagement, a significant increase in the amount of the receptor is observed in high-density DRMs. Similarly to CD16b, CD11b also resides in low- and high-density DRMs. In contrast with CD16b, the partitioning of CD11b in DRMs does not change in response to CD16b engagement. We also provide evidence for the implication of Syk in CD16b signalling and its partitioning to DRMs in resting and activated PMNs (polymorphonuclear neutrophils). Additionally, DRM-disrupting agents, such as nystatin and methyl-β-cyclodextrin, alter cellular responses to CD16b receptor ligation. Notably, a significant increase in the mobilization of intracellular Ca^{2+} and in tyrosine phosphorylation of intracellular substrates after CD16b engagement is observed. Altogether, the results of this study provide evidence that high-density DRMs play a role in CD16b signalling in human neutrophils.

3.720 Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis
Abrami, L., Leppla, S.H., Gisou van der Gaat, F.
J. Cell Biol., ***171(2)***, *309-320 (2006)*

The anthrax toxin is composed of three independent polypeptide chains. Successful intoxication only occurs when heptamerization of the receptor-binding polypeptide, the protective antigen (PA), allows binding of the two enzymatic subunits before endocytosis. We show that this tailored behavior is caused by two counteracting posttranslational modifications in the cytoplasmic tail of PA receptors. The receptor is palmitoylated, and this unexpectedly prevents its association with lipid rafts and, thus, its premature ubiquitination. This second modification, which is mediated by the E3 ubiquitin ligase Cbl, only occurs in rafts and is required for rapid endocytosis of the receptor. As a consequence, cells expressing palmitoylation-defective mutant receptors are less sensitive to anthrax toxin because of a lower number of surface receptors as well as premature internalization of PA without a requirement for heptamerization.

3.721 Deactivation of phosporylated and nonphosphorylated rhodopsin by arrestin splice variants
Burns, M.E. et al
J. Neurosci., ***26(3)***, *1036-1044 (2006)*

Arrestins constitute a family of small cytoplasmic proteins that mediate deactivation of G-protein-coupled receptors (GPCRs) and are known to be essential for cascade inactivation and receptor desensitization. Alternative splicing produces an array of arrestin gene products that have widely different specificities for their cognate receptors *in vitro*, but the differential functions of these splice variants *in vivo* are essentially unknown. Bovine rod photoreceptors express two splice variants of visual arrestin (p44 and p48) that display different affinities for the GPCR rhodopsin. To determine the functions of these splice variants in intact cells, we expressed a transgene encoding either a truncated form of murine arrestin ($mArr^{1–369}$, or m44) or the long (p48) isoform in mouse rods lacking endogenous arrestin (Arr–/–). Morphological analysis showed that expression of either variant attenuated the light-induced degeneration that is thought to result from excessive cascade activity in Arr–/–rods. Suction electrode recordings from individual rods indicated that the expression of either m44 or p48 splice variants could restore normal kinetics to Arr–/– dim flash responses, indicating that both isoforms can bind to and quench phosphorylated rhodopsin rapidly. To our surprise, only the full-length variant was able to alter the kinetics of responses in rods lacking both arrestin and rhodopsin kinase, indicating that p48 can also quench the activity of nonphosphorylated rhodopsin.

3.722 LMP1 signaling and activation of NF-κB LMP1 transgenic mice
Thornburg, N.J. et al
*Oncogene, **25**, 288-297 (2006)*

Transgenic mice expressing Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1) under the control of an immunoglobulin heavy-chain promoter and enhancer develop lymphoma at a threefold higher incidence than LMP1-negative mice. *In vitro*, LMP1 activates numerous signaling pathways including p38, c-Jun N terminal kinase (JNK), phosphatidylinositol 3 kinase (PI3K)/Akt, and NF-κB through interactions with tumor necrosis receptor-associated factors (TRAFs). These pathways are frequently activated in EBV-associated malignancies, although their activation cannot be definitively linked to LMP1 expression *in vivo*.

In this study, interactions between LMP1 and TRAFs and the activation of PI3K/Akt, JNK, p38, and NF-κB were examined in LMP1 transgenic mice. LMP1 co-immunoprecipitated with TRAFs 1, 2, and 3. Akt, JNK, and p38 were activated in LMP1-positive and -negative splenocytes as well as LMP1-positive and -negative lymphomas. Multiple forms of NF-κB were activated in healthy splenocytes from LMP1 transgenic mice, in contrast to healthy splenocytes from LMP1-negative mice. However, in both LMP1-positive and -negative lymphomas, only the oncogenic NF-κB c-Rel, was specifically activated. Similarly to EBV-associated malignancies, p53 protein was detected at high levels in the transgenic lymphomas, although mutations were not detected in the p53 gene. These data indicate that NF-κB is activated in LMP1-positive healthy splenocytes; however, NF-κB c-Rel is specifically activated in both the transgenic lymphomas and in the rare lymphomas that develop in negative mice. The LMP1-mediated activation of NF-κB may contribute to the specific activation of c-Rel and lead to the increased development of lymphoma in the LMP1 transgenic mice.

3.723 Intracytoplasmic maturation of the human immunodeficiency virus type I reverse transcription complexes determines their capacity to integrate into chromatin
Iodanskiy, S., Berro, R., Altieri, M., Kashanchi, F. And Bukrinsky, M.
*Retrovirology, **3(4)**, 1-12 (2006)*

Background
The early events of the HIV-1 life cycle include entry of the viral core into target cell, assembly of the reverse transcription complex (RTCs) performing reverse transcription, its transformation into integration-competent complexes called pre-integration complexes (PICs), trafficking of complexes into the nucleus, and finally integration of the viral DNA into chromatin. Molecular details and temporal organization of these processes remain among the least investigated and most controversial problems in the biology of HIV.
Results
To quantitatively evaluate maturation and nuclear translocation of the HIV-1 RTCs, nucleoprotein complexes isolated from the nucleus (nRTC) and cytoplasm (cRTC) of HeLa cells infected with MLV Env-pseudotyped HIV-1 were analyzed by real-time PCR. While most complexes completed reverse transcription in the cytoplasm, some got into the nucleus before completing DNA synthesis. The HIV-specific RNA complexes could get into the nucleus when reverse transcription was blocked by reverse

transcriptase inhibitor, although nuclear import of RNA complexes was less efficient than of DNA-containing RTCs. Analysis of the RTC nuclear import in synchronized cells infected in the G2/M phase of the cell cycle showed enrichment in the nuclei of RTCs containing incomplete HIV-1 DNA compared to non-synchronized cells, where RTCs with complete reverse transcripts prevailed. Immunoprecipitation assays identified viral proteins IN, Vpr, MA, and cellular Ini1 and PML associated with both cRTCs and nRTCs, whereas CA was detected only in cRTCs and RT was diminished in nRTCs. Cytoplasmic maturation of the complexes was associated with increased immunoreactivity with anti-Vpr and anti-IN antibodies, and decreased reactivity with antibodies to RT. Both cRTCs and nRTCs carried out endogenous reverse transcription reaction *in vitro*. In contrast to cRTCs, *in vitro* completion of reverse transcription in nRTCs did not increase their integration into chromatin.
Conclusion
These results suggest that RTC maturation occurs predominantly in the cytoplasm. Immature RTCs containing RT and incomplete DNA can translocate into the nucleus during mitosis and complete reverse transcription, but are defective for integration.

3.724 Glycosylphosphatidylinositol-anchored proteins are required for the transport pg detergent-resistant microdomain-associated membrane proteins Tat2p and Fur4p
Okamoto, M., Yoko-o, T., Umemura, M., Nakayama, K-i. and Jigami, Y.
J. Biol. Chem., ***281(7)***, *4013-4023 (2006)*

In eukaryotic cells many cell surface proteins are attached to the membrane via the glycosylphosphatidylinositol (GPI) moiety. In yeast, GPI also plays important roles in the production of mannoprotein in the cell wall. We previously isolated *gwt1* mutants and found that *GWT1* is required for inositol acylation in the GPI biosynthetic pathway. In this study we isolated a new *gwt1* mutant allele, *gwt1-10*, that shows not only high temperature sensitivity but also low temperature sensitivity. The *gwt1-10* cells show impaired acyltransferase activity and attachment of GPI to proteins even at the permissive temperature. We identified *TAT2*, which encodes a high affinity tryptophan permease, as a multicopy suppressor of cold sensitivity in *gwt1-10* cells. The *gwt1-10* cells were also defective in the import of tryptophan, and a lack of tryptophan caused low temperature sensitivity. Microscopic observation revealed that Tat2p is not transported to the plasma membrane but is retained in the endoplasmic reticulum in *gwt1-10* cells grown under tryptophan-poor conditions. We found that Tat2p was not associated with detergent-resistant membranes (DRMs), which are required for the recruitment of Tat2p to the plasma membrane. A similar result was obtained for Fur4p, a uracil permease localized in the DRMs of the plasma membrane. These results indicate that GPI-anchored proteins are required for the recruitment of membrane proteins Tat2p and Fur4p to the plasma membrane via DRMs, suggesting that some membrane proteins are redistributed in the cell in response to environmental and nutritional conditions due to an association with DRMs that is dependent on GPI-anchored proteins.

3.725 Involvement of lipid rafts and caveolae in cardiac ion channel function
Maguy, A., Hebert, T.E. and Nattel, S.
Cardiovasc. Res., ***69(4)***, *798-807 (2006)*

A variety of lipid microdomains, including caveolae, have been shown to play an important role in both protein targetting and in controlling protein–protein interactions. There is increasing evidence for significant ion channel localization in lipid rafts. Cardiac channel subunits known to localize in lipid rafts include Kv1.4, Kv1.5, Kv2.1, Kv4, Kir2, Kir3, K_{ATP}, Nav and Cav subunits. This article reviews what is known about the occurrence and functional significance of cardiac ion channel/lipid raft interactions. Much remains to be learned about this area of potentially enormous importance to cardiac function in health and disease.

3.726 Notch, epidermal growth factor receptor, and β1-integrin pathways are coordinated in neural stem cells
Campos, L., Decker, L., Taylor, V. and Skarnes, W.
J. Biol. Chem., ***281(8)***, *5300-5309 (2006)*

Notch1 and β1-integrins are cell surface receptors involved in the recognition of the niche that surrounds stem cells through cell-cell and cell-extracellular matrix interactions, respectively. Notch1 is also involved in the control of cell fate choices in the developing central nervous system (Lewis, J. (1998) *Semin. Cell Dev. Biol.* 9, 583-589). Here we report that Notch and β1-integrins are co-expressed and that these proteins cooperate with the epidermal growth factor receptor in neural progenitors. We describe data that suggests

that β1-integrins may affect Notch signaling through 1) physical interaction (sequestration) of the Notch intracellular domain fragment by the cytoplasmic tail of the β1-integrin and 2) affecting trafficking of the Notch intracellular domain via caveolin-mediated mechanisms. Our findings suggest that caveolin 1-containing lipid rafts play a role in the coordination and coupling of β1-integrin, Notch1, and tyrosine kinase receptor signaling pathways. We speculate that this will require the presence of the adequate β1-activating extracellular matrix or growth factors in restricted regions of the central nervous system and namely in neurogenic niches.

3.727 **Association of brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2) with recycling endosomes during transferring uptake**
Shen, X. et al
*PNAS, **103(8)**, 2635-2640 (2006)*

ADP-ribosylation factors (ARFs) are critical in vesicular trafficking. Brefeldin A-inhibited guanine nucleotide-exchange protein (BIG)1 and BIG2 activate ARFs by accelerating replacement of bound GDP with GTP. Additional and differing functions of these ≈200-kDa proteins are now being recognized, as are their independent intracellular movements. Here, we describe the localization in COS7 cells by immunofluorescence microscopy of BIG2, but not BIG1, with structures that have characteristics of recycling endosomes during transferrin (Tfn) uptake and Tfn receptor (TfnR) recycling. Cell content of BIG2 and Rab11, but not TfnR, BIG1, Rab4, or Exo70, was increased after 60 min of Tfn uptake. BIG2, but not BIG1, appeared in density-gradient fractions containing TfnR, Rab11, and Exo70 after 60 min of Tfn uptake. Treatment of cells with BIG2 small interfering RNA (siRNA), but not BIG1 or control siRNAs, decreased BIG2 protein >90% without affecting BIG1, ARF, or actin content, whereas TfnR was significantly increased as was its accumulation in perinuclear recycling endosomes. Tfn release appeared unaffected by BIG1 siRNA but was significantly slowed from cells treated with BIG2 siRNA alone or plus BIG1 siRNA. We suggest that BIG2 has an important role in Tfn uptake and TfnR recycling, perhaps through its demonstrated interaction with Exo70 and the exocyst complex.

3.728 **The HIV lipodome: a raft with an unusual composition**
Brügger, B. et al
*PNAS, **103(8)**, 2641-2646 (2006)*

The lipids of enveloped viruses play critical roles in viral morphogenesis and infectivity. They are derived from the host membranes from which virus budding occurs, but the precise lipid composition has not been determined for any virus. Employing mass spectrometry, this study provides a quantitative analysis of the lipid constituents of HIV and a comprehensive comparison with its host membranes. Both a substantial enrichment of the unusual sphingolipid dihydrosphingomyelin and a loss of viral infectivity upon inhibition of sphingolipid biosynthesis in host cells are reported, establishing a critical role for this lipid class in the HIV replication cycle. Intriguingly, the overall lipid composition of native HIV membranes resembles detergent-resistant membrane microdomains and is strikingly different from that of host cell membranes. With this composition, the HIV lipidome provides strong evidence for the existence of lipid rafts in living cells.

3.729 **Vesicle-associated membrane protein 7 is expressed in intestinal ER**
Siddiqi, S., Mathan, J., Siddiqi, S., Gorelick, F.S. and Mansbach, C.M.
*J. Cell Sci., **119**, 943-950 (2006)*

Intestinal dietary triacylglycerol absorption is a multi-step process. Triacylglycerol exit from the endoplasmic reticulum (ER) is the rate-limiting step in the progress of the lipid from its apical absorption to its basolateral membrane export. Triacylglycerol is transported from the ER to the cis Golgi in a specialized vesicle, the pre-chylomicron transport vesicle (PCTV). The vesicle-associated membrane protein 7 (VAMP7) was found to be more concentrated on PCTVs compared with ER membranes. VAMP7 has been previously identified associated with post-Golgi sites in eukaryotes. To examine the potential role of VAMP7 in PCTV trafficking, antibodies were generated that identified a 25 kDa band consistent with VAMP7 but did not crossreact with VAMP1,2. VAMP7 was concentrated on intestinal ER by immunofluorescence microscopy. Immunoelectron microscopy showed that the ER proteins Sar1 and rBet1 were present on PCTVs and colocalized with VAMP7. **Iodixanol** gradient centrifugation showed VAMP7 to be isodense with ER and endosomes. Although VAMP7 localized to intestinal ER, it was not present in

the ER of liver and kidney. Anti-VAMP7 antibodies reduced the transfer of triacylglycerol, but not newly synthesized proteins, from the ER to the Golgi by 85%. We conclude that VAMP7 is enriched in intestinal ER and that it plays a functional role in the delivery of triacylglycerol from the ER to the Golgi.

3.730 Conditioned medium from enterohemorrhagic *Escherichia coli*-infected T84 cells inhibits signal transducer and activator of transcription 1 activation by gamma interferon
Jandu, N. Et al
*Infect. Immun., **74(3)**, 1809-1818 (2006)*

Gamma interferon (IFN-γ) is a cytokine important to host defense which can signal through signal transducer and activator of transcription 1 (Stat1). Enterohemorrhagic *Escherichia coli* (EHEC) modulates host cell signal transduction to establish infection, and EHEC serotypes O113:H21 and O157:H7 both inhibit IFN-γ-induced Stat1 tyrosine phosphorylation in vitro. The aim of this study was to delineate both bacterial and host cell factors involved in the inhibition of Stat1 tyrosine phosphorylation. Human T84 colonic epithelial cells were challenged with direct infection, viable EHEC separated from T84 cells by a filter, sodium orthovanadate, isolated flagellin, bacterial culture supernatants, and conditioned medium treated with proteinase K, trypsin, or heat inactivation. Epithelial cells were then stimulated with IFN-γ and protein extracts were analyzed by immunoblotting. The data showed that IFN-γ-inducible Stat1 tyrosine phosphorylation was inhibited when EHEC adhered to T84 cells, but not by bacterial culture supernatants or bacteria separated from the epithelial monolayer. Conditioned medium from T84 cells infected with EHEC O157:H7 suppressed Stat1 activation, and this was not reversed by treatment with proteinases or heat inactivation. Use of pharmacological inhibitors showed that time-dependent bacterial, but not epithelial, protein synthesis was involved. Stat1 inhibition was also independent of bacterial flagellin, host proteasome activity, and protein tyrosine phosphatases. Infection led to altered IFN-γ receptor domain 1 subcellular distribution and decreased expression in cholesterol-enriched membrane microdomains. Thus, suppression of host cell IFN-γ signaling by production of a contact-dependent, soluble EHEC factor may represent a novel mechanism for this pathogen to evade the host immune system.

3.731 The association of Shiga-like toxin with detergent-resistant membranes is modulated by glucosylceramide and is an essential requirement in the endoplasmic reticulum for a cytotoxic effect
Smith, D.C. et al
*Mol. Biol. Cell, **17**, 1375-1387 (2006)*

Receptor-mediated internalization to the endoplasmic reticulum (ER) and subsequent retro-translocation to the cytosol are essential sequential processes required for the productive intoxication of susceptible mammalian cells by Shiga-like toxin-1 (SLTx). Recently, it has been proposed that the observed association of certain ER-directed toxins and viruses with detergent-resistant membranes (DRM) may provide a general mechanism for their retrograde transport to endoplasmic reticulum (ER). Here, we show that DRM recruitment of SLTx bound to its globotriosylceramide (Gb_3) receptor is mediated by the availability of other glycosphingolipids. Reduction in glucosylceramide (GlcCer) levels led to complete protection against SLTx and a reduced cell surface association of bound toxin with DRM. This reduction still allowed efficient binding and transport of the toxin to the ER. However, toxin sequestration within DRM of the ER was abolished under reduced GlcCer conditions, suggesting that an association of toxin with lipid microdomains or rafts in the ER (where these are defined by detergent insolubility) is essential for a later step leading to or involving retro-translocation of SLTx across the ER membrane. In support of this, we show that a number of ER residents, proteins intimately involved in the process of ER dislocation of misfolded proteins, are present in DRM.

3.732 Cytokine receptor-mediated trafficking of preformed IL-4 in eosinophils identifies an innate immune mechanism of cytokine secretion
Spencer, L.A. et al
*PNAS, **103(9)**, 3333-3338 (2006)*

Although leukocytes of the innate immune system, including eosinophils, contain within their granules preformed stores of cytokines available for selective and rapid release, little is known about the mechanisms governing the mobilization and secretion of these cytokines. Here we show that a cytokine receptor, the IL-4 receptor α chain, mediates eotaxin-stimulated mobilization of preformed IL-4 from eosinophil granules into secretory vesicles. Eosinophils contain substantial intracellular quantities of several granule- and vesicle-associated cytokine receptors, including IL-4, IL-6, and IL-13 receptors as

well as CCR3. Both IL-4 and IL-4 receptor α chain colocalized in eosinophil granules; and after eotaxin-stimulation, IL-4 receptor α chain, bearing bound IL-4, was mobilized into secretory vesicles. These findings indicate that intracellular cytokine receptors within secretory vesicles transport their cognate cytokines requisite for the secretion of cytokines preformed in innate immune leukocytes.

3.733 Glial cell line-derived neurotrophic factor-dependent recruitment of ret into lipid rafts enhances signaling by partitioning ret from proteasome-dependent degradation
Pierchala, B.A., Milbrandt, J. and Johnson Jr., E.M.
J. Neurosci., ***26(10)****, 2777-2787 (2006)*

The receptor tyrosine kinase (RTK) Ret is activated by the formation of a complex consisting of ligands such as glial cell line-derived neurotrophic factor (GDNF) and glycerophosphatidylinositol-anchored coreceptors termed GFRαs. During activation, Ret translocates into lipid rafts, which is critical for functional responses to GDNF. We found that Ret was rapidly ubiquitinated and degraded in sympathetic neurons when activated with GDNF, but, unlike other RTKs that are trafficked to lysosomes for degradation, Ret was degraded predominantly by the proteasome. After GDNF stimulation, the majority of ubiquitinated Ret was located outside of lipid rafts and Ret was lost predominantly from nonraft membrane domains. Consistent with the predominance of Ret degradation outside of rafts, disruption of lipid rafts in neurons did not alter either the GDNF-dependent ubiquitination or degradation of Ret. GDNF-mediated survival of sympathetic neurons was inhibited by lipid raft depletion, and this inhibitory effect of raft disruption on GDNF-mediated survival was reversed if Ret degradation was blocked via proteasome inhibition. Therefore, lipid rafts sequester Ret away from the degradation machinery located in nonraft membrane domains, such as Cbl family E3 ligases, thereby sustaining Ret signaling.

3.734 Sorting of Pmel17 to melanosomes through the plasma membrane by AP1 and AP2: evidence for the polarized nature of melanocytes
Valencia, J.C. et al
J. Cell Sci., ***119****, 1080-1091 (2006)*

Adaptor proteins (AP) play important roles in the sorting of proteins from the trans-Golgi network, but how they function in the sorting of various melanosome-specific proteins such as Pmel17, an essential structural component of melanosomes, in melanocytes is unknown. We characterized the processing and trafficking of Pmel17 via adaptor protein complexes within melanocytic cells. Proteomics analysis detected Pmel17, AP1 and AP2, but not AP3 or AP4 in early melanosomes. Real-time PCR, immunolabeling and tissue in-situ hybridization confirmed the coexpression of AP1 isoforms μ1A and μ1B (expressed only in polarized cells) in melanocytes and keratinocytes, but expression of μ1B is missing in some melanoma cell lines. Transfection with AP1 isoforms (μ1A or μ1B) showed two distinct distribution patterns that involved Pmel17, and only μ1B was able to restore the sorting of Pmel17 to the plasma membrane in cells lacking μ1B expression. Finally, we established that expression of μ1B is regulated physiologically in melanocytes by UV radiation or DKK1. These results show that Pmel17 is sorted to melanosomes by various intracellular routes, directly or indirectly through the plasma membrane, and the presence of basolateral elements in melanocytes suggests their polarized nature.

3.735 Multifunctional analysis of *Chlamydia*-specific genes in a yeast expression system
Sisko, J.L., Spaeth, K., Kumar, Y. And Valdivia, R.H.
Mol. Microbiol., ***60(1)****, 51-66 (2006)*

Our understanding of how obligate intracellular pathogens co-opt eukaryotic cellular functions has been limited by their intractability to genetic manipulation and by the abundance of pathogen-specific genes with no known functional homologues. In this report we describe a gene expression system to characterize proteins of unknown function from the obligate intracellular bacterial pathogen *Chlamydia trachomatis*. We have devised a homologous recombination-based cloning strategy to construct an ordered array of *Saccharomyces cerevisiae* strains expressing all *Chlamydia*-specific genes. These strains were screened to identify chlamydial proteins that impaired various yeast cellular functions or that displayed tropism towards eukaryotic organelles. In addition, to identify bacterial factors that are secreted into the host cell, recombinant chlamydial proteins were screened for reactivity towards antisera raised against vacuolar membranes purified from infected mammalian cells. We report the identification of 34 *C. trachomatis* proteins that impact yeast cellular functions or are tropic for a range of eukaryotic organelles including mitochondria, nucleus and cytoplasmic lipid droplets, and a new family of *Chlamydia*-specific proteins that are exported from the parasitopherous vacuole. The versatility of molecular manipulations and protein

expression in yeast allows for the rapid construction of comprehensive protein expression arrays to explore the function of pathogen-specific gene products from microorganisms that are difficult to genetically manipulate, grow in culture or too dangerous for routine analysis in the laboratory.

3.736 Rescue of cell growth by sphingosine with disruption of lipid microdomain formation in *Saccharomyces cerevisiae* deficient in sphingolipid biosynthesis
Tani, M., Khara, A. and Igarashi, Y.
Biochem. J., ***394***, *237-242 (2006)*

In the yeast *Saccharomyces cerevisiae*, sphingolipids are essential for cell growth. Inactivation of sphingolipid biosynthesis, such as by disrupting the serine palmitoyltransferase gene (*LCB2*), is lethal, but cells can be rescued by supplying an exogenous LCB (long-chain base) like PHS (phytosphingosine) or DHS (dihydrosphingosine). In the present study, supplying SPH (sphingosine), an unnatural LCB for yeast, similarly rescued the Δ*lcb2* cells, but only when SPH 1-phosphate production was inhibited by deleting the LCB kinase gene *LCB4*. Exogenously added SPH was adequately converted into phosphoinositol-containing complex sphingolipids. Interestingly, cells carrying SPH-based sphingolipids exhibited a defect in the association of Pma1p with Triton X-100-insoluble membrane fractions, and displayed sensitivities to both Ca^{2+} and hygromycin B. These results suggest that the SPH-based sphingolipids in these cells have properties that differ from those of the PHS- or DHS-based sphingolipids in regard to lipid microdomain formation, leading to abnormal sensitivities towards certain environmental stresses. The present paper is the first report showing that in sphingolipid-deficient *S. cerevisiae*, the requirement for LCB can be fulfilled by exogenous SPH, although this supplement results in failure of lipid microdomain formation.

3.737 Localization of a portion of the liver isoform of fatty-acid-binding protein (L-FABP) to peroxisomes
Antonenkov, V.D. et al
Biochem. J., ***394***, *475-484 (2006)*

The liver isoform of fatty-acid-binding protein (L-FABP) facilitates the cellular uptake, transport and metabolism of fatty acids and is also involved in the regulation of gene expressions and cell differentiation. Consistent with these functions, L-FABP is predominantly present in the cytoplasm and to a lesser extent in the nucleus; however, a significant portion of this protein has also been detected in fractions containing different organelles. More recent observations, notably on L-FABP-deficient mice, indicated a possible direct involvement of L-FABP in the peroxisomal oxidation of long-chain fatty acids. In order to clarify the links between L-FABP and peroxisomal lipid metabolism, we reinvestigated the subcellular distribution of the protein. Analytical subcellular fractionation by a method preserving the intactness of isolated peroxisomes, two-dimensional gel electrophoresis of peroxisomal matrix proteins combined with MS analysis, and immunoelectron microscopy of liver sections demonstrate the presence of L-FABP in the matrix of peroxisomes as a soluble protein. Peroxisomal L-FABP was highly inducible by clofibrate. The induction of L-FABP was accompanied by a marked increase in the binding capacity of peroxisomal matrix proteins for oleic acid and *cis*-parinaric acid. The peroxisomal β-oxidation of palmitoyl-CoA and acyl-CoA thioesterase activity were stimulated by L-FABP, indicating that the protein modulates the function of peroxisomal lipid-metabolizing enzymes. The possible role of intraperoxisomal L-FABP in lipid metabolism is discussed.

3.738 Internalized Pseudomonas exotoxin A can exploit multiple pathways to reach the endoplasmic reticulum
Smith, D.C. et al
Traffic, ***7(4)***, *379-393 (2006)*

Receptor-mediated internalization to the endoplasmic reticulum (ER) and subsequent retro-translocation to the cytosol are essential sequential processes required for the intoxication of mammalian cells by Pseudomonas exotoxin A (PEx). The toxin binds the α2-macroglobulin receptor/low-density lipoprotein receptor-related protein. Here, we show that in HeLa cells, PEx recruits a proportion of this receptor to detergent-resistant microdomains (DRMs). Uptake of receptor-bound PEx involves transport steps both directly from early endosomes to the *trans*-Golgi network (TGN) independently of Rab9 function and from late endosomes to the TGN in a Rab9-dependent manner. Furthermore, treatments that simultaneously perturb both Arf1-dependent and Rab6-dependent retrograde pathways show that PEx can use multiple routes to reach the ER. The Rab6-dependent route has only been described previously for cargo with lipid-sorting signals. These findings suggest that partial localization of PEx within DRM permits a choice of

trafficking routes consistent with a model that DRM-associated toxins reach the ER on a lipid-dependent sorting pathway whilst non-DRM-associated PEx exploits the previously characterized KDEL receptor-mediated uptake pathway. Thus, unexpectedly, an ER-directed toxin with a proteinaceous receptor shows promiscuity in its intracellular trafficking pathways, exploiting routes controlled by both lipid- and protein-sorting signals.

3.739 Dissecting Rotavirus Particle-Raft Interaction with Small Interfering RNAs: Insights into Rotavirus Transit through the Secretory Pathway
Cuadras, M.A., Bordier, B.B., Zambrano, J.L., Ludert, J.E. and Greenberg, H.B.
J. Virol., ***80(8)***, *3935-3946 (2006)*

Studies of rotavirus morphogenesis, transport, and release have shown that although these viruses are released from the apical surface of polarized intestinal cells before cellular lysis, they do not follow the classic exocytic pathway. Furthermore, increasing evidence suggests that lipid rafts actively participate in the exit of rotavirus from the infected cell. In this study, we silenced the expression of VP4, VP7, and NSP4 by using small interfering RNAs (siRNAs) and evaluated the effect of shutting down the expression of these proteins on rotavirus-raft interactions. Silencing of VP4 and NSP4 reduced the association of rotavirus particles with rafts; in contrast, inhibition of VP7 synthesis slightly affected the migration of virions into rafts. We found that inhibition of rotavirus migration into lipid rafts, by either siRNAs or tunicamycin, also specifically blocked the targeting of VP4 to rafts, suggesting that the association of VP4 with rafts is mostly mediated by the formation of viral particles in the endoplasmic reticulum (ER). We showed that two populations of VP4 exist, one small population that is independently targeted to rafts and a second large pool of VP4 whose association with rafts is mediated by particle formation in the ER. We also present evidence to support the hypothesis that assembly of VP4 into mature virions takes place in the late stages of transit through the ER. Finally, we analyzed the progression of rotavirus proteins in the exocytic pathway and found that VP4 and virion-assembled VP7 colocalized with ERGIC-53, suggesting that rotavirus particles transit through the intermediate compartment between the ER and the Golgi complex.

3.740 Rab1 Defines a Novel Pathway Connecting the Pre-Golgi Intermediate Compartment with the Cell Periphery
Sannerud, R. et al
Mol. Biol. Cell, ***17***, *1514-1526 (2006)*

The function of the pre-Golgi intermediate compartment (IC) and its relationship with the endoplasmic reticulum (ER) and Golgi remain only partially understood. Here, we report striking segregation of IC domains in polarized PC12 cells that develop neurite-like processes. Differentiation involves expansion of the IC and movement of Rab1-containing tubules to the growth cones of the neurites, whereas p58- and COPI-positive IC elements, like rough ER and Golgi, remain in the cell body. Exclusion of Rab1 effectors p115 and GM130 from the neurites further indicated that the centrifugal, Rab1-mediated pathway has functions that are not directly related to ER-to-Golgi trafficking. Disassembly of COPI coats did not affect this pathway but resulted in missorting of p58 to the neurites. Live cell imaging showed that green fluorescent protein (GFP)–Rab1A-containing IC elements move bidirectionally both within the neurites and cell bodies, interconnecting different ER exit sites and the *cis*-Golgi region. Moreover, in nonpolarized cells GFP-Rab1A-positive tubules moved centrifugally towards the cell cortex. Hydroxymethylglutaryl-CoA reductase, the key enzyme of cholesterol biosynthesis, colocalized with slowly sedimenting, Rab1-enriched membranes when the IC subdomains were separated by velocity sedimentation. These results reveal a novel pathway directly connecting the IC with the cell periphery and suggest that this Rab1-mediated pathway is linked to the dynamics of smooth ER.

3.741 Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by peptide-linked anti-CD30-auristatin conjugates
Kung, M.S. et al
J. Biol. Chem., ***281(15)***, *10540-10547 (2006)*

The chimeric anti-CD30 monoclonal antibody cAC10, linked to the antimitotic agents monomethyl auristatin E (MMAE) or F (MMAF), produces potent and highly CD30-selective anti-tumor activity *in vitro* and *in vivo*. These drugs are appended via a valine-citrulline (vc) dipeptide linkage designed for high stability in serum and conditional cleavage and putative release of fully active drugs by lysosomal cathepsins. To characterize the biochemical processes leading to effective drug delivery, we examined the intracellular trafficking, internalization, and metabolism of the parent antibody and two antibody-drug

conjugates, cAC10vc-MMAE and cAC10vc-MMAF, following CD30 surface antigen interaction with target cells. Both cAC10 and its conjugates bound to target cells and internalized in a similar manner. Subcellular fractionation and immunofluorescence studies demonstrated that the antibody and antibody-drug conjugates entering target cells migrated to the lysosomes. Trafficking of both species was blocked by inhibitors of clathrin-mediated endocytosis, suggesting that drug conjugation does not alter the fate of antibody-antigen complexes. Incubation of cAC10vc-MMAE or cAC10vc-MMAF with purified cathepsin B or with enriched lysosomal fractions prepared by subcellular fractionation resulted in the release of active, free drug. Cysteine protease inhibitors, but not aspartic or serine protease inhibitors, blocked antibody-drug conjugate metabolism and the ensuing cytotoxicity of target cells and yielded enhanced intracellular levels of the intact conjugates. These findings suggest that in addition to trafficking to the lysosomes, cathepsin B and perhaps other lysosomal cysteine proteases are requisite for drug release and provide a mechanistic basis for developing antibody-drug conjugates cleavable by intracellular proteases for the targeted delivery of anti-cancer therapeutics.

3.742 Reproducibility of LC-MS-based protein identification
Berg, M., Parbel, A., Pettersen, H., Fenyo, D. and Björkesten, L.
J. Exp. Botany, ***57(7)***, *1509-1514 (2006)*

Traditional analysis of liquid chromatography-mass spectrometry (LC-MS) data, typically performed by reviewing chromatograms and the corresponding mass spectra, is both time-consuming and difficult. Detailed data analysis is therefore often omitted in proteomics applications. When analysing multiple proteomics samples, it is usually only the final list of identified proteins that is reviewed. This may lead to unnecessarily complex or even contradictory results because the content of the list of identified proteins depends heavily on the conditions for triggering the collection of tandem mass spectra. Small changes in the signal intensity of a peptide in different LC-MS experiments can lead to the collection of a tandem mass spectrum in one experiment but not in another. Also, the quality of the tandem mass spectrometry experiments can vary, leading to successful identification in some cases but not in others. Using a novel image analysis approach, it is possible to achieve repeat analysis with a very high reproducibility by matching peptides across different LC-MS experiments using the retention time and parent mass over charge (*m/z*). It is also easy to confirm the final result visually. This approach has been investigated by using tryptic digests of integral membrane proteins from organelle-enriched fractions from *Arabidopsis thaliana* and it has been demonstrated that very highly reproducible, consistent, and reliable LC-MS data interpretation can be made.

3.743 The nuclear microspherule protein 58 is a novel RNA-binding protein that interacts with fragile X mental retardation in polyribosomal mRNPs from neurons
Davidovic, L. et al
Hum. Mol. Genet., ***15(9)***, *1525-1538 (2006)*

The fragile X syndrome, the leading cause of inherited mental retardation, is due to the inactivation of the fragile mental retardation 1 gene (*FMR1*) and the subsequent absence of its gene product FMRP. This RNA-binding protein is thought to control mRNA translation and its absence in fragile X cells leads to alteration in protein synthesis. In neurons, FMRP is thought to repress specific mRNAs during their transport as silent ribonucleoparticles (mRNPs) from the cell body to the distant synapses which are the sites of local synthesis of neuro-specific proteins. The mechanism by which FMRP sorts out its different mRNAs targets might be tuned by the intervention of different proteins. Using a yeast two-hybrid system, we identified MicroSpherule Protein 58 (MSP58) as a novel FMRP-cellular partner. In cell cultures, we found that MSP58 is predominantly present in the nucleus where it interacts with the nuclear isoform of FMRP. However, in neurons but not in glial cells, MSP58 is also present in the cytoplasmic compartment, as well as in neurites, where it co-localizes with FMRP. Biochemical evidence is given that MSP58 is associated with polyribosomal poly(A)+ mRNPs. We also show that MSP58, similar to FMRP, is present on polyribosomes prepared from synaptoneurosomes and that it behaves as an RNA-binding protein with a high affinity to the G-quartet structure. We propose that this novel cellular partner for FMRP escorts FMRP-containing mRNP from the nucleus and nucleolus to the somato-dendritic compartment where it might participate in neuronal translation regulation.

3.744 Overloading of stable and exclusion of unstable human superoxide dismutase-1 variants in mitochondria of murine amyotrophic lateral sclerosis models
Bergemalm, D. et al
J. Neurosci., ***26(16)***, *4147-4154 (2006)*

Mutants of human superoxide dismutase-1 (hSOD1) cause amyotrophic lateral sclerosis (ALS), and mitochondria are thought to be primary targets of the cytotoxic action. The high expression rates of hSOD1s in transgenic ALS models give high levels of the stable mutants G93A and D90A as well as the wild-type human enzyme, significant proportions of which lack Cu and the intrasubunit disulfide bond. The endogenous murine SOD1 (mSOD1) also lacks Cu and is disulfide reduced but is active and oxidized in mice expressing the low-level unstable mutants G85R and G127insTGGG. The possibility that the molecular alterations may cause artificial loading of the stable hSOD1s into mitochondria was explored. Approximately 10% of these hSOD1s were localized to mitochondria, reaching levels 100-fold higher than those of mSOD1 in control mice. There was no difference between brain and spinal cord and between stable mutants and the wild-type hSOD1. mSOD1 was increased fourfold in mitochondria from high-level hSOD1 mice but was normal in those with low levels, suggesting that the Cu deficiency and disulfide reduction cause mitochondrial overloading. The levels of G85R and G127insTGGG mutant hSOD1s in mitochondria were 100- and 1000-fold lower than those of stable mutants. Spinal cords from symptomatic mice contained hSOD1 aggregates covering the entire density gradient, which could contaminate isolated organelle fractions. Thus, high hSOD1 expression rates can cause artificial loading of mitochondria. Unstable low-level hSOD1s are excluded from mitochondria, indicating other primary locations of injury. Such models may be preferable for studies of ALS pathogenesis.

3.745 Cytoplasmic tails of SialT2 and GalNacT impose their respective proximal and distal Golgi localization

Uliana, A.S., Giraudo, C.G. and Maccioni, H.J.F.
Traffic, ***7***, *604-612 (2006)*

Complex glycolipid synthesis is catalyzed by different glycosyltransferases resident of the Golgi complex. Most of them are type II membrane proteins comprising a lumenal, C-terminal domain linked to an N-terminal domain (Ntd) constituted by a short cytoplasmic tail (ct), a transmembrane, and a lumenal stem regions. They concentrate selectively in different sub-Golgi compartments, in an overlapped manner, acting in succession in the addition of sugars to acceptor glycolipids. The Ntds are sufficient to localize glycosyltransferases in the Golgi complex, but it is not clear whether they also confer selective concentration in sub-Golgi compartments. Here, we studied whether the Ntd of SialT2, localized in the proximal Golgi, and the one of GalNAcT, a *trans*/TGN Golgi-concentrated enzyme, concentrate reporter proteins in the corresponding sub-Golgi compartment. The sub-Golgi concentration of the Ntds fused to spectral variants of the GFP was determined in CHO-K1 cells from their behavior upon addition of brefeldin A. Fluorescence microscopy and subcellular fractionation showed that the SialT2 Ntd concentrates in a proximal sub-Golgi compartment – and that of GalNAcT in TGN elements. Exchanging the transmembrane region and the cts of SialT2 and GalNAcT indicates that information for proximal or distal Golgi concentration is associated with the cts.

3.746 rAAV2 traffics through both the late and the recycling endosomes in a dose-dependent fashion

Ding, W., Zhang, L.N., Yeaman, C. and Engelhardt, J.F.
Mol. Ther., ***13(4)***, *671-682 (2006)*

Inefficient trafficking of recombinant adeno-associated virus type-2 (rAAV2) to the nucleus is a major barrier for transduction. Using imaging and subcellular fractionation techniques, we evaluated the extent of rAAV2 movement through the late (Rab7) and recycling (Rab11) endosomes. Following rAAV2 infection of HeLa cells, immunoisolation of HA–Rab7- or HA–Rab11-tagged endosomes and intracellular colocalization of Cy3-labeled rAAV2 with EGFP–Rab7 or EGFP–Rab11 markers demonstrated dose-dependent trafficking of rAAV2 through the recycling and late endosomal compartments. At low multiplicities of infection (m.o.i. 100 genomes/cell), rAAV2 predominantly trafficked to the Rab7 compartment. In contrast, rAAV2 predominantly trafficked to the recycling endosome at 100-fold higher m.o.i. siRNA studies inhibiting either Rab7 or Rab11 demonstrated that reducing Rab11 protein levels more significantly inhibited rAAV2 transduction on a per genome basis compared to inhibition of Rab7. Dose–response curves, comparing the m.o.i. of AV2Luc infection to relative transduction, also supported the hypothesis that viral movement through the Rab11 compartment at high m.o.i. is more competent for transgene expression ($\sim$100-fold) than virus that moves through the Rab7 compartment at low m.o.i. These findings suggest that strategies to shunt viral movement from the late to the recycling endosome may be effective at increasing viral transduction for gene therapy.

3.747 Mapping the *Arabidopsis* organelle proteome
Dunkley, T.P.J. et al
*PNAS, **103(17)**, 6518-6523 (2006)*

A challenging task in the study of the secretory pathway is the identification and localization of new proteins to increase our understanding of the functions of different organelles. Previous proteomic studies of the endomembrane system have been hindered by contaminating proteins, making it impossible to assign proteins to organelles. Here we have used the localization of organelle proteins by the isotope tagging technique in conjunction with isotope tags for relative and absolute quantitation and 2D liquid chromatography for the simultaneous assignment of proteins to multiple subcellular compartments. With this approach, the density gradient distributions of 689 proteins from *Arabidopsis thaliana* were determined, enabling confident and simultaneous localization of 527 proteins to the endoplasmic reticulum, Golgi apparatus, vacuolar membrane, plasma membrane, or mitochondria and plastids. This parallel analysis of endomembrane components has enabled protein steady-state distributions to be determined. Consequently, genuine organelle residents have been distinguished from contaminating proteins and proteins in transit through the secretory pathway.

3.748 Opposite effect of caveolin-1 in the metabolism of high-density and low-density lipoproteins
Truong, T.Q., Aubin, D., Bourgeois, P., Falstrault, L. and Brisette, L.
*Biochim. Biophys. Acta, **1761**, 24-36 (2006)*

Receptors of the scavenger class B family were reported to be localized in caveolae, the cell surface microdomains rich in free cholesterol and glycosphyngolipids, which are characterized by the presence of caveolin-1. Parenchymal hepatic and hepatoma HepG2 cells express very low levels of caveolin-1. In the present study, stable transformants of HepG2 cells expressing caveolin-1 were generated to address the effect of caveolin-1 on receptor activity. Compared to normal cells, these cells show higher ^{125}I-bovine serum albumin (BSA) uptake and cholesterol efflux, two indicators of functional caveolae. By immunoprecipitation, cell fractionation and confocal analyses, we found that caveolin-1 is well colocalized with the cluster of differentiation-36 (CD36) and the low-density lipoprotein (LDL) receptor (LDLr) but to a lesser extent with the scavenger receptor class B type I (SR-BI) in HepG2 cells expressing caveolin-1. However, caveolin-1 expression favors the dimerization of SR-BI. Two clones of cells expressing caveolin-1 were investigated for their lipoprotein metabolism activity. Compared to normal cells, these cells show a 71–144% increase in ^{125}I-LDL degradation. The analysis of the cholesteryl esters (CE)-selective uptake (CE association minus protein association) revealed that the expression of caveolin-1 in HepG2 cells decreases by 59%–73% LDL-CE selective uptake and increases high-density lipoprotein (HDL)-CE selective uptake by 44%–66%. We conclude that the expression of caveolin-1 in HepG2 cells moves the balance of LDL degradation/CE selective uptake towards degradation and favors HDL-CE selective uptake. Thus, in the normal hepatic parenchymal situation where caveolin-1 is poorly expressed, LDL-CE selective uptake is the preferred pathway.

3.749 Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization
Verma, R. et al
*J. Clin. Invest., **116(5)**, 1346-1359 (2006)*

A properly established and maintained podocyte intercellular junction, or slit diaphragm, is a necessary component of the selective permeability barrier of the kidney glomerulus. The observation that mutation or deletion of the slit diaphragm transmembrane protein nephrin results in failure of podocyte foot process morphogenesis and concomitant proteinuria first suggested the hypothesis that nephrin serves as a component of a signaling complex that directly integrates podocyte junctional integrity with cytoskeletal dynamics. The observations made herein provide the first direct evidence to our knowledge for a phosphorylation-mediated signaling mechanism by which this integrative function is derived. Our data support the model that during podocyte intercellular junction formation, engagement of the nephrin ectodomain induces transient Fyn catalytic activity that results in nephrin phosphorylation on specific nephrin cytoplasmic domain tyrosine residues. We found that this nephrin phosphorylation event resulted in recruitment of the SH2–SH3 domain–containing adapter protein Nck and assembly of actin filaments in an Nck-dependent fashion. Considered in the context of the role of nephrin family proteins in other organisms and the integral relationship of actin dynamics and junction formation, these observations establish a function for nephrin in regulating actin cytoskeletal dynamics.

3.750 The Interaction between Cytoplasmic Prion Protein and the Hydrophobic Lipid Core of Membrane Correlates with Neurotoxicity

Wang, X., Wang, F., Arterburn, L., Wollmann, R. and Ma, J.
J. Biol. Chem., ***281(19)***, *13559-13565 (2006)*

Prion protein (PrP), normally a cell surface protein, has been detected in the cytosol of a subset of neurons. The appearance of PrP in the cytosol could result from either retro-translocation of misfolded PrP from the endoplasmic reticulum (ER) or impaired import of PrP into the ER. Transgenic mice expressing cytoplasmic PrP (cyPrP) developed neurodegeneration in cerebellar granular neurons, although no detectable pathology was observed in other brain regions. In order to understand why granular neurons in the cerebellum were most susceptible to cyPrP-induced degeneration, we investigated the subcellular localization of cyPrP. Interestingly, we found that cyPrP is membrane-bound. In transfected cells, it binds to the ER and plasma/endocytic vesicular membranes. In transgenic mice, it is associated with synaptic and microsomal membranes. Furthermore, the cerebellar neurodegeneration in transgenic mice correlates with the interaction between cyPrP and the hydrophobic lipid core of the membrane but not with either the aggregation status or the dosage of cyPrP. These results suggest that lipid membrane perturbation could be a cellular mechanism for cyPrP-induced neurotoxicity and explain the seemingly conflicting results concerning cyPrP.

3.751 Differential expression of nuclear AT1 receptors and angiotensin II within the kidney of the male congenic mRen2.Lewis rat

Pendergrass, K.D., Averill, D.B., Ferrario, C.M., Diz, D.I. and Chappell, M.C.
Am. J. Physiol. Renal Physiol., ***290***, *F1497-F1506 (2006)*

We established a new congenic model of hypertension, the mRen(2).Lewis rat and assessed the intracellular expression of angiotensin peptides and receptors in the kidney. The congenic strain was established from the backcross of the (mRen2)27 transgenic rat that expresses the mouse renin 2 gene onto the Lewis strain. The 20-wk-old male congenic rats were markedly hypertensive compared with the Lewis controls (systolic blood pressure: 195 ± 2 vs. 107 ± 2 mmHg, $P < 0.01$). Although plasma ANG II levels were not different between strains, circulating levels of ANG-(1–7) were 270% higher and ANG I concentrations were 40% lower in the mRen2.Lewis rats. In contrast, both cortical (CORT) and medullary (MED) ANG II concentrations were 60% higher in the mRen2.Lewis rats, whereas tissue ANG I was 66 and 84% lower in CORT and MED. For both strains, MED ANG II, ANG I, and ANG-(1–7) were significantly higher than CORT levels. Intracellular ANG II binding distinguished nuclear (NUC) and plasma membrane (PM) receptor using the ANG II radioligand ^{125}I-sarthran. Isolated CORT nuclei exhibited a high density (B_{max} >200 fmol/mg protein) and affinity for the sarthran ligand (K_D<0.5 nM); the majority of these sites (>95%) were the AT_1 receptor subtype. CORT ANG II receptor B_{max} and K_D values in nuclei were 75 and 50% lower, respectively, for the mRen2.Lewis vs. the Lewis rats. In the MED, the PM receptor density (Lewis: 50 ± 4 vs. mRen2.Lewis: 21 ± 5 fmol/mg protein) and affinity (Lewis: 0.31 ± 0.1 vs. 0.69 ± 0.1 nM) were lower in the mRen2.Lewis rats. In summary, the hypertensive mRen2.Lewis rats exhibit higher ANG II in both CORT and MED regions of the kidney. Evaluation of intracellular ANG II receptors revealed lower CORT NUC and MED PM AT_1 sites in the mRen2.Lewis. The downregulation of AT_1 sites in the mRen2.Lewis rats may reflect a compensatory response to dampen the elevated levels of intrarenal ANG II.

3.752 MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion

Spinazzola, A. et al
Nature Genetics, ***38(5)***, *570-575 (2006)*

The mitochondrial (mt) DNA depletion syndromes (MDDS) are genetic disorders characterized by a severe, tissue-specific decrease of mtDNA copy number, leading to organ failure. There are two main clinical presentations: myopathic (OMIM 609560) and hepatocerebral1 (OMIM 251880). Known mutant genes, including TK2 (ref. 2), SUCLA2 (ref. 3), DGUOK (ref. 4) and POLG 5, 6, account for only a fraction of MDDS cases7. We found a new locus for hepatocerebral MDDS on chromosome 2p21-23 and prioritized the genes on this locus using a new integrative genomics strategy. One of the top-scoring candidates was the human ortholog of the mouse kidney disease gene Mpv17 (ref. 8). We found disease-segregating mutations in three families with hepatocerebral MDDS and demonstrated that, contrary to the alleged peroxisomal localization of the MPV17 gene product9, MPV17 is a mitochondrial inner membrane

protein, and its absence or malfunction causes oxidative phosphorylation (OXPHOS) failure and mtDNA depletion, not only in affected individuals but also in Mpv17-/- mice.

3.753 Gas1 Is Related to the Glial Cell-derived Neurotrophic Factor Family Receptors αand Regulates Ret Signaling
Cabrera, J.R. et al
J. Biol. Chem., ***281(20)***, *14330-14339 (2006)*

The growth arrest-specific gene 1 (Gas1) protein has been proposed to function during development as an inhibitor of growth and a mediator of cell death and is also re-expressed in adult neurons during excitotoxic insult. Here we have demonstrated that the Gas1 protein shows high structural similarity to the glial cell-derived neurotrophic factor (GDNF) family receptors α, which mediate GDNF responses through the receptor tyrosine kinase Ret. We found that Gas1 binds Ret in a ligand-independent manner and sequesters Ret in lipid rafts. Signaling downstream of Ret is thus modified through a mechanism that involves the adaptor protein Shc as well as ERK, eventually blocking Akt activation. Consequently, when Gas1 is induced, Ret-mediated GDNF-dependent survival effects are compromised. The growth arrest-specific gene 1 (Gas1) protein has been proposed to function during development as an inhibitor of growth and a mediator of cell death and is also re-expressed in adult neurons during excitotoxic insult. Here we have demonstrated that the Gas1 protein shows high structural similarity to the glial cell-derived neurotrophic factor (GDNF) family receptors α, which mediate GDNF responses through the receptor tyrosine kinase Ret. We found that Gas1 binds Ret in a ligand-independent manner and sequesters Ret in lipid rafts. Signaling downstream of Ret is thus modified through a mechanism that involves the adaptor protein Shc as well as ERK, eventually blocking Akt activation. Consequently, when Gas1 is induced, Ret-mediated GDNF-dependent survival effects are compromised.

3.754 Sequence Requirements for Localization of Human Cytomegalovirus Tegument Protein pp28 to the Virus Assembly Compartment and for Assembly of Infectious Virus
Seo, J-Y. and Britt, W.J.
J. Virol., ***80(11)***, *5611-5626 (2006)*

The human cytomegalovirus UL99 open reading frame encodes a 190-amino-acid (aa) tegument protein, pp28, that is myristoylated and phosphorylated. pp28 is essential for assembly of infectious virus, and nonenveloped virions accumulate in the cytoplasm of cells infected with recombinant viruses with a UL99 deletion. pp28 is localized to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) in transfected cells, while in infected cells, it is localized together with other virion proteins in a juxtanuclear compartment termed the assembly compartment (AC). We investigated the sequence requirements for pp28 trafficking to the AC and assembly of infectious virus. Our studies indicated that the first 30 to 35 aa were required for localization of pp28 to the ERGIC in transfected cells. Mutant forms of pp28 containing only the first 35 aa localized with other virion structural proteins to cytoplasmic compartments early in infection, but localization to the AC at late times required a minimum of 50 aa. In agreement with previous reports, we demonstrated that the deletion of a cluster of acidic amino acids (aa 44 to 59) prevented wild-type trafficking of pp28 and recovery of infectious virus. A recombinant virus expressing only the first 50 aa was replication competent, and this mutant, pp28, localized to the AC in cells infected with this virus. These findings argued that localization of pp28 to the AC was essential for assembly of infectious virus and raised the possibility that amino acids in the amino terminus of pp28 have additional roles in the envelopment and assembly of the virion other than simply localizing pp28 to the AC.

3.755 Detergent-free caveolae proteome suggests an interaction with ER and mitochondria
McMahon, K-A. et al
Proteomics, ***6***, *143-152 (2006)*

Recent proteomic studies of detergent resistant membrane fractions have begun to characterize the protein composition of caveolae and lipid rafts. The methods used in most of these studies, however, are not able to distinguish between plasma membrane and internal membrane lipid domains. Here we used a non-detergent method for obtaining fractions enriched in caveolae derived from the plasma membrane of multiple cell types. Unexpectedly, the proteins in the caveolae proteome suggest these lipid domains may interact with elements of ER and mitochondria. A comparison of the partial proteome we obtained with other published reports identifies 26 proteins that are candidate marker proteins for identifying caveolae in multiple cell types.

3.756 Deletion of SERP1/RAMP4, a Component of the Endoplasmic Reticulum (ER) Translocation Sites, Leads to ER Stress
Hori, O. et al
Mol. Cell. Biol., ***26(11)****, 4257-4267 (2006)*

Stress-associated endoplasmic reticulum (ER) protein 1 (SERP1), also known as ribosome-associated membrane protein 4 (RAMP4), is a Sec61-associated polypeptide that is induced by ER stress. SERP1$^{-/-}$ mice, made by targeted gene disruption, demonstrated growth retardation, increased mortality, and impaired glucose tolerance. Consistent with high levels of SERP1 expression in pancreas, pancreatic islets from SERP1$^{-/-}$ mice failed to rapidly synthesize proinsulin in response to a glucose load. In addition, reduced size and enhanced ER stress were observed in the anterior pituitary of SERP1$^{-/-}$ mice, and growth hormone production was slowed in SERP1$^{-/-}$ pituitary after insulin stimulation. Experiments using pancreatic microsomes revealed aberrant association of ribosomes and the Sec61 complex and enhanced ER stress in SERP1$^{-/-}$ pancreas. In basal conditions, the Sec61 complex in SERP1$^{-/-}$ microsomes was more cofractionated with ribosomes, compared with SERP1$^{+/+}$ counterparts, in high-salt conditions. In contrast, after glucose stimulation, the complex showed less cofractionation at an early phase (45 min) but more at a later phase (120 min). Although intracellular insulin/proinsulin levels were not significantly changed in both genotypes, these results suggest that subtle changes in translocation efficiency play an important role in the regulation of ER stress and rapid polypeptide synthesis.

3.757 DHCR24-Knockout Embryonic Fibroblasts Are Susceptible to Serum Withdrawal-Induced Apoptosis Because of Dysfunction of Caveolae and Insulin-Akt-Bad Signaling
Lu, X. et al
Endocrinology, ***147(6)****, 3123-3132 (2006)*

The DHCR24 gene encodes an enzyme catalyzing the last step of cholesterol biosynthesis, the conversion of desmosterol to cholesterol. To elucidate the physiological significance of cholesterol biosynthesis in mammalian cells, we investigated proliferation of mouse embryonic fibroblasts (MEFs) prepared from DHCR24$^{-/-}$ mice. Both DHCR24$^{-/-}$ and wild-type MEFs proliferated in the presence of serum in culture media. However, the inhibition of external cholesterol supply by serum withdrawal induced apoptosis of DHCR24$^{-/-}$ MEFs, which was associated with a marked decrease in the intracellular and plasma membrane cholesterol levels, Akt inactivation, and Bad dephosphorylation. Insulin is an antiapoptotic factor capable of stimulating the Akt-Bad cascade, and its receptor (IR) is enriched in caveolae, cholesterol-rich microdomains of plasma membrane. We thus analyzed the association of IR and caveolae in the cholesterol-depleted MEFs. Subcellular fractionation and immunocytochemical analyses revealed that the IR and caveolin-1 contents were markedly reduced in the caveolae fraction of the MEFs, suggesting the disruption of caveolae, and that large amounts of IR were present apart from caveolin-1 on plasma membrane, indicating the uncoupling of IR with caveolae. Consistent with these findings, insulin-dependent phosphorylations of insulin receptor substrate-1, Akt, and Bad were impaired in the cholesterol-depleted MEFs. However, this impairment was partial because treatment of the MEFs with insulin restored Akt activation and prevented apoptosis. Cholesterol supply also prevented apoptosis. These results demonstrate that the cellular cholesterol biosynthesis is critical for the activation and maintenance of the Akt-Bad cell survival cascade in response to growth factors such as insulin.

3.758 Membrane vesicles shed by *Legionella pneumophila* inhibit fusion of phagosomes with lysosomes
Fernandez-Moreira, E., Helbig, J.H. and Swanson, M.S.
*Infect. Immun.,****74(6)****, 3285-3295 (2006)*

When cultured in broth to the transmissive phase, *Legionella pneumophila* infects macrophages by inhibiting phagosome maturation, whereas replicative-phase cells are transported to the lysosomes. Here we report that the ability of *L. pneumophila* to inhibit phagosome-lysosome fusion correlated with developmentally regulated modifications of the pathogen's surface, as judged by its lipopolysaccharide profile and by its binding to a sialic acid-specific lectin and to the hydrocarbon hexadecane. Likewise, the composition of membrane vesicles shed by *L. pneumophila* was developmentally regulated, based on binding to the lectin and to the lipopolysaccharide-specific monoclonal antibody 3/1. Membrane vesicles were sufficient to inhibit phagosome-lysosome fusion by a mechanism independent of type IV secretion, since only ~25% of beads suspended with or coated by vesicles from transmissive phase wild type or *dotA* secretion mutants colocalized with lysosomal probes, whereas ~75% of beads were lysosomal when untreated or presented with vesicles from the *L. pneumophila letA* regulatory mutant or *E. coli*. As

observed previously for *L. pneumophila* infection of mouse macrophages, vesicles inhibited phagosome-lysosome fusion only temporarily; by 10 h after treatment with vesicles, macrophages delivered ~72% of ingested beads to lysosomes. Accordingly, in the context of the epidemiology of the pneumonia Legionnaires' disease and virulence mechanisms of *Leishmania* and *Mycobacteria*, we discuss a model here in which *L. pneumophila* developmentally regulates its surface composition and releases vesicles into phagosomes that inhibit their fusion with lysosomes.

3.759 Mutational analysis of the Lem3p-Dnf1p putative phospholipid-translocating P-type ATPase reveals novel regulatory roles for Lem3p and a carboxyl-terminal region of Dnf1p independent of the phospholipid-translocating activity of Dnf1p in yeast
Noji, T. et al
Biochem. Biophys. Res. Comm., ***344***, *323-331 (2006)*

Lem3p-Dnf1p is a putative aminophospholipid translocase (APLT) complex that is localized to the plasma membrane; Lem3p is required for Dnf1p localization to the plasma membrane. We have identified *lem3* mutations, which did not affect formation or localization of the Lem3p-Dnf1p complex, but caused a synthetic growth defect with the null mutation of *CDC50*, a structurally and functionally redundant homologue of *LEM3*. Interestingly, these *lem3* mutants exhibited nearly normal levels of NBD-labeled phospholipid internalization across the plasma membrane, suggesting that Lem3p may have other functions in addition to regulation of the putative APLT activity of Dnf1p at the plasma membrane. Similarly, deletion of the COOH-terminal cytoplasmic region of Dnf1p affected neither the localization nor the APLT activity of Dnf1p at the plasma membrane, but caused a growth defect in the *cdc50Δ* background. Our results suggest that the Lem3p-Dnf1p complex may play a role distinct from its plasma membrane APLT activity when it substitutes for the Cdc50p-Drs2p complex, its redundant partner in the endosomal/*trans*-Golgi network compartments.

3.760 The rab exchange factor Sec2p reversibly associates with the exocyst
Medkova, M., France, Y.E., Coleman, J. and Novick, P.
Mol. Biol. Cell, ***17(6)****, 2757-2769 (2006)*

Activation of the rab GTPase, Sec4p, by its exchange factor, Sec2p, is needed for polarized transport of secretory vesicles to exocytic sites and for exocytosis. A small region in the C-terminal half of Sec2p regulates its localization. Loss of this region results in temperature-sensitive growth and the depolarized accumulation of secretory vesicles. Here, we show that Sec2p associates with the exocyst, an octameric effector of Sec4p involved in tethering secretory vesicles to the plasma membrane. Specifically, the exocyst subunit Sec15p directly interacts with Sec2p. This interaction normally occurs on secretory vesicles and serves to couple nucleotide exchange on Sec4p to the recruitment of the Sec4p effector. The mislocalization of Sec2p mutants correlates with dramatically enhanced binding to the exocyst complex. We propose that Sec2p is normally released from the exocyst after vesicle tethering so that it can recycle onto a new round of vesicles. The mislocalization of Sec2p mutants results from a failure to be released from Sec15p, blocking this recycling pathway.

3.761 Caveolin-1 is required for fatty acid translocase (FAT/CD36) localization and function at the plasma membrane of mouse embryonic fibroblasts
Ring, A., Le Lay, S., pohl, J., Verkade, P. and Stremmel, W.
Biochim. Biophys. Acta, ***1761****, 416-423 (2006)*

Several lines of evidence suggest that lipid rafts are involved in cellular fatty acid uptake and influence fatty acid translocase (FAT/CD36) function. However, it remains unknown whether caveolae, a specialized raft type, are required for this mechanism. Here, we show that wild-type (WT) mouse embryonic fibroblasts (MEFs) and caveolin-1 knockout (KO) MEFs, which are devoid of caveolae, have comparable overall expression of FAT/CD36 protein but altered subcellular FAT/CD36 localization and function. In WT MEFs, FAT/CD36 was isolated with both lipid raft enriched detergent-resistant membranes (DRMs) and detergent-soluble membranes (DSMs), whereas in cav-1 KO cells it was exclusively associated with DSMs. Subcellular fractionation demonstrated that FAT/CD36 in WT MEFs was localized intracellularly and at the plasma membrane level while in cav-1 KO MEFs it was absent from the plasma membrane. This mistargeting of FAT/CD36 in cav-1 KO cells resulted in reduced fatty acid uptake compared to WT controls. Adenoviral expression of caveolin-1 in KO MEFs induced caveolae formation, redirection of FAT/CD36 to the plasma membrane and rescue of fatty acid uptake. In conclusion, our data provide

evidence that caveolin-1 is necessary to target FAT/CD36 to the plasma membrane. Caveolin-1 may influence fatty acid uptake by regulating surface availability of FAT/CD36.

3.762 Phosphorylation of phototransduction GAP RGS9-1 depends on the α isoform of protein kinase C
Wang, Q., Hu, G., Leitges, M. and Wensel, T.G.
Invest. Ophthalmol. Vis. Sci., ***47***, *E-abstract 822 (2006)*

Purpose: To investigate the regulation of the photoreceptor–specific GTPase Accelerating Protein (GAP) RGS9–1 by PKCα. Normal light response kinetics require both RGS9–1 and its membrane anchor R9AP, whose interactions have been previously shown to be modified by a light–inhibited phosphorylation on Ser^{475} of RGS9–1 catalyzed by one or more isozymes of protein kinase C (PKC).
Methods: PKCα–/– and wild–type mice were compared. The presence of PKCα in mouse rod outer segments was examined by western blot of purified rod outer segments. The localization of PKCα to rod outer segments was confirmed by analysis of fractions collected from two successive sucrose gradients and iso–osmotic **OptiPrep** gradient purification. The amount of PKCα in purified intact rod outer segments was determined by quantitative western blot of rod outer segments and retina lysates. *In vitro* phosphorylation of RGS9–1 in rod outer segments by endogenous PKCα was detected by kinase assays. *In vivo* light–regulated phosphorylation of RGS9–1 at Ser^{475} was assessed by immunoprecipitation of RGS9–1 from mice retinas and followed by western blot using Ser^{475}–phosphate–specific antibody.
Results: PKCα immuno–reactivity is present in wild–type mouse rod outer segments but missing in those of PKCα –/– mice. It consistently co–purifies with rod outer segments. Approximately 20% of the total retinal PKCα pool is in mouse rod outer segments. PKCα knockouts do not exhibit the light–sensitive phosphorylation of RGS9–1 on PKC target site Ser^{475} that is observed in wild–type mice and show slower RGS9–1 Ser^{475} phosphorylation *in vitro*.
Conclusion: PKCα immuno–reactivity in photoreceptor cells is genuine and not due to cross–activity and contamination. PKCα is essential for light–sensitive RGS9–1 Ser^{475} phosphorylation. These results indicate that rod outer segments contain modest levels of PKCα, and this enzyme is responsible for regulation of the R9AP–RGS9–1 membrane complex by Ser^{475} phosphorylation, thus likely regulating the rate–limiting reaction in vision.

3.763 Regulation of RPE phagocytosis by integrin receptor-tetraspanin surface membrane domains
Finnemann, S.C. and Chang, Y.
Invest. Ophthalmol. Vis. Sci., ***47***, *e-abstract 5879 (2006)*

Purpose: Retinal pigment epithelial (RPE) cells depend on αvß5 integrin receptors and their downstream signaling pathways to maximize their phagocytic activity following circadian photoreceptor outer segment (POS) shedding in the retina. We hypothesize that the activity of αvß5 integrin itself may be regulated in the RPE to synchronize phagocytosis. Integrin receptors may associate with proteins of the tetraspanin family in specialized membrane microdomains to control integrin signaling. Here, we test whether tetraspanins functionally interact with αvß5 integrin in RPE cells. Methods: RT–PCRs and immunoblotting established tetraspanin expression in RPE cells *in vitro* and *in vivo*. We used confocal microscopy following live cell labeling with receptor antibodies and choleratoxin B to co–localize αvß5 integrin and tetraspanins to plasma membrane sub–domains at the apical surface of the RPE. We used **optiprep** gradient fractionation of RPE cell lysates and co–immunoprecipitation assays to further test subcellular co–fractionation of tetraspanins and αvß5 integrin. We used isolated POS fragments in quantitative uptake assays to determine effects of surface cholesterol depletion, tetraspanin antibody blocking and tetraspanin overexpression on the phagocytic function of RPE cells in culture. **Results:** We found that tetraspanins CD9, CD63 and CD81 localize to the apical, phagocytic surface of RPE cells in culture. CD81 partially co–localized with αvß5 integrin. Furthermore, we identified CD81 in a complex with αvß5 integrin in RPE cells in culture and in intact retina. CD81 co–fractionated with αvß5 in low–density membrane domains of RPE cell lysates. Cholesterol depletion, which altered CD81 and αvß5 surface distribution, as well as specific inhibition of CD81 reduced POS phagocytosis by human and rat RPE cell cultures. In contrast, CD81 overexpression increased POS phagocytosis. **Conclusions:** Our results demonstrate that the apical plasma membrane tetraspanin CD81 regulates the phagocytic activity of the RPE. Functional interaction with tetraspanins including CD81 and microdomain recruitment may contribute to the temporal control of αvß5 integrin receptor signaling in the RPE that is necessary for rhythmic phagocytosis of shed POS. Ongoing experiments study CD81 and integrin αvß5 complex formation and distribution during active RPE phagocytosis *in vitro* and *in vivo*.

3.764 Lipid raft-based membrane compartmentation of a plant transport protein expressed in *Saccharomyces cerevisiae*

Grossmann, G., Opekarova, M., Novakova, L., Stolz, J. and Tanner, W.
*Eukaryot. Cell, **5(6)**, 945-953 (2006)*

The hexose-proton symporter HUP1 shows a spotty distribution in the plasma membrane of the green alga *Chlorella kessleri*. *Chlorella* cannot be transformed so far. To study the membrane localization of the HUP1 protein in detail, the symporter was fused to green fluorescent protein (GFP) and heterologously expressed in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In these organisms, the HUP1 protein has previously been shown to be fully active. The GFP fusion protein was exclusively targeted to the plasma membranes of both types of fungal cells. In *S. cerevisiae*, it was distributed nonhomogenously and concentrated in spots resembling the patchy appearance observed previously for endogenous H^+ symporters. It is documented that the *Chlorella* protein colocalizes with yeast proteins that are concentrated in 300-nm raft-based membrane compartments. On the other hand, it is completely excluded from the raft compartment housing the yeast H^+/ATPase. As judged by their solubilities in Triton X-100, the HUP1 protein extracted from *Chlorella* and the GFP fusion protein extracted from *S. cerevisiae* are detergent-resistant raft proteins. *S. cerevisiae* mutants lacking the typical raft lipids ergosterol and sphingolipids showed a homogenous distribution of HUP1-GFP within the plasma membrane. In an ergosterol synthesis (*erg6*) mutant, the rate of glucose uptake was reduced to less than one-third that of corresponding wild-type cells. In *S. pombe*, the sterol-rich plasma membrane domains can be stained in vivo with filipin. *Chlorella* HUP1-GFP accumulated exactly in these domains. Altogether, it is demonstrated here that a plant membrane protein has the property of being concentrated in specific raft-based membrane compartments and that the information for its raft association is retained between even distantly related organisms.

3.765 Assembly of infectious HIV-1 in human epithelial and T-lymphoblastic cell lines

Grigorov, B., Arcanger, F., Roingeard, P., Darlix, J-L. and Muriaux, D.
*J. Mol. Biol., **359**, 848-862 (2006)*

The canonical view of the ultimate steps of HIV-1 replication is that virus assembly and budding are taking place at the plasma membrane of infected cells. Surprisingly, recent studies revealed that these steps also occur on endosomal membranes in the interior of infected cells, such as macrophages. This prompted us to revisit the site of HIV-1 assembly in human epithelial-like cells and in infected human T-lymphoblastic cells. To address this question, we investigated the intracellular location of the major viral structural components of HIV-1, namely Gag, Env and the genomic RNA. Using a sub-cellular fractionation method, as well as immuno-confocal and electron microscopy, we show that Gag, the Env glycoproteins and the genomic RNA accumulate in late endosomes that contain infectious HIV-1 particles. In epithelial-like 293T cells, HIV-1 assembles and buds both at the plasma membrane and in endosomes, while in chronically infected human T lymphocytes, viral assembly mostly occurs within the cell where large amounts of infectious virions accumulate in endosomal compartments. In addition, HIV-1 release could be enhanced by ionomycin, a drug stimulating calcium-dependent exocytosis. These results favour the view that newly made Gag molecules associate with the genomic RNA in the cytosol, then viral core complexes can be targeted to late endosomes together with Env, where infectious HIV-1 are made and subsequently released by exocytosis.

3.766 Thyroid hormone receptor isoforms localize to cardiac mitochondrial matrix with potential for binding to receptor elements on mtDNA

Morrish, F. et al
*Mitochondrion, **6(3)**, 143-148 (2006)*

Thyroid hormone (T_3) rapidly promotes both nuclear and mitochondrial DNA transcription in cardiomyocytes, suggesting that T3 directly binds and activates mitochondrial genes. We showed for the first time mitochondrial localization for multiple TRα isoforms in heart, including truncated versions. Additionally, we demonstrated novel mitochondrial localization for versions of $TR\alpha_2$, the dominant negative isoform lacking a functional ligand-binding domain. We also confirmed by electromobility shift assays, that $TR\alpha_2$ in mitochondrial extracts binds to thyroid receptor response elements present in the 12S rRNA (DRO) and D-loop region (DR2) of mitochondrial DNA. Thus, TRα isoforms may directly regulate T_3 responses at mtDNA in the heart.

3.767 Intracellular ATP-sensitive K^+ channels in mouse pancreata beta cells: against a role in organelle cation homeostasis
Varadi, A. et al
*Diabetologia, **49**, 1567-1577 (2006)*

Aims/hypothesis ATP-sensitive K^+ (K_{ATP}) channels located on the beta cell plasma membrane play a critical role in regulating insulin secretion and are targets for the sulfonylurea class of antihyperglycaemic drugs. Recent reports suggest that these channels may also reside on insulin-containing dense-core vesicles and mitochondria. The aim of this study was to explore these possibilities and to test the hypothesis that vesicle-resident channels play a role in the control of organellar Ca^{2+} concentration or pH.
Methods To quantify the subcellular distribution of the pore-forming subunit Kir6.2 and the sulfonylurea binding subunit SUR1 in isolated mouse islets and clonal pancreatic MIN6 beta cells, we used four complementary techniques: immunoelectron microscopy, density gradient fractionation, vesicle immunopurification and fluorescence-activated vesicle isolation. Intravesicular and mitochondrial concentrations of free Ca^{2+} were measured in intact or digitonin-permeabilised MIN6 cells using recombinant, targeted aequorins, and intravesicular pH was measured with the recombinant fluorescent probe pHluorin.
Results SUR1 and Kir6.2 immunoreactivity were concentrated on dense-core vesicles and on vesicles plus the endoplasmic reticulum/Golgi network, respectively, in both islets and MIN6 cells. Reactivity to neither subunit was detected on mitochondria. Glibenclamide, tolbutamide and diazoxide all failed to affect Ca^{2+} uptake into mitochondria, and K_{ATP} channel regulators had no significant effect on intravesicular free Ca^{2+} concentrations or vesicular pH.
Conclusions/Interpretation A significant proportion of Kir6.2 and SUR1 subunits reside on insulin-secretory vesicles and the distal secretory pathway in mouse beta cells but do not influence intravesicular ion homeostasis. We propose that dense-core vesicles may serve instead as sorting stations for the delivery of channels to the plasma membrane.

3.768 Cholesterol depletion facilitates ubiquitylation of NPC1 and its association with SKD1/Vps4
Ohsaki, Y. et al
*J. Cell Sci., **119**, 2643-2653 (2006)*

Niemann-Pick disease type C (NPC) is an inherited lipid storage disorder caused by mutations in *NPC1* or *NPC2*. NPC1 is a polytopic glycoprotein that contains a sterol-sensing domain, whereas NPC2 is a soluble protein that contains an MD-2-like lipid-recognition domain. In the current study, we addressed the hypothesis that ubiquitylation of NPC1 might be regulated by cholesterol. We found that depletion of cellular cholesterol facilitated ubiquitylation of NPC1 expressed in COS cells. A loss-of-function mutant, NPC1(P691S), which contains an amino acid substitution in the sterol-sensing domain, failed to respond to cholesterol depletion. Another mutant, NPC1(δLLNF), which lacks the endosomal-targeting motif, also failed to respond. SKD1(E235Q), a dominant-negative mutant of SKD1/Vps4 that inhibits disassembly of the endosomal sorting complex required for transport (ESCRT), caused an accumulation of ubiquitylated NPC1. SKD1(E235Q) associated with NPC1 on the endosomal membrane, whereas wild-type SKD1 associated with NPC1 only when cells were depleted of cholesterol. Similarly, in control human skin fibroblasts, cholesterol depletion facilitated ubiquitylation of endogenous NPC1. In patient cells that lack NPC2 function, NPC1 was ubiquitylated regardless of cellular cholesterol levels, suggesting that NPC2 is required to prevent NPC1 ubiquitylation under cholesterol-rich conditions. These results suggest that ubiquitylation of NPC1 and its association with the ESCRT complex are controlled by endosomal cholesterol levels utilizing a mechanism that involves NPC2.

3.769 A rat model of human FENIB (familial encephalopathy with neuroserpin inclusion bodies)
Takano, K. et al
*Biochim. Biophys. Res. Comm, **346(3)**, 1040-1047 (2006)*

FENIB (familial encephalopathy with neuroserpin inclusion bodies) is caused by intracellular accumulation/polymerization of mutant neuroserpins in the endoplasmic reticulum (ER). Transgenic rats overexpressing megsin (Tg meg), a newly identified serine protease inhibitor (serpin), demonstrated intraneuronal periodic–acid Schiff (PAS)-positive inclusions distributed throughout deeper layers of cerebral cortex, CA1 of the hippocampus, and substantia nigra. Hippocampal extracts from Tg meg rats showed increased expression of ER stress proteins, and activation of caspases-12 and -3, associated with decreased neuronal density. Enhanced ER stress was also observed in dopaminergic neurons in the substantia nigra, in parallel with decreased neuronal viability and motor coordination. In each case, PAS-

positive inclusions were also positive for megsin. These data suggest that overexpression of megsin results in ER stress, eventuating in the formation of PAS-positive inclusions. Tg meg rats provide a novel model of FENIB, where accumulation of serpins in the ER induces selective dysfunction/loss of specific neuronal populations.

3.770 Retrovirus infection strongly enhances scrapie infectivity release in cell culture
Leblanc, P. et al
*EMBO J., **25**, 2674-2685 (2006)*

Prion diseases are neurodegenerative disorders associated in most cases with the accumulation in the central nervous system of PrP^{Sc} (conformationally altered isoform of cellular prion protein (PrP^{C}); Sc for scrapie), a partially protease-resistant isoform of the PrP^{C}. PrP^{Sc} is thought to be the causative agent of transmissible spongiform encephalopathies. The mechanisms involved in the intercellular transfer of PrP^{Sc} are still enigmatic. Recently, small cellular vesicles of endosomal origin called exosomes have been proposed to contribute to the spread of prions in cell culture models. Retroviruses such as murine leukemia virus (MuLV) or human immunodeficiency virus type 1 (HIV-1) have been shown to assemble and bud into detergent-resistant microdomains and into intracellular compartments such as late endosomes/multivesicular bodies. Here we report that moloney murine leukemia virus (MoMuLV) infection strongly enhances the release of scrapie infectivity in the supernatant of coinfected cells. Under these conditions, we found that PrP^{C}, PrP^{Sc} and scrapie infectivity are recruited by both MuLV virions and exosomes. We propose that retroviruses can be important cofactors involved in the spread of the pathological prion agent.

3.771 Impairment of mitochondrial anti-oxidant defence in SOD1-related motor neuron injury and amelioration by ebselen
Wood-Allum, C.A. et al
*Brain, **129**, 1693-1709 (2006)*

There is now compelling evidence of mitochondrial dysfunction in motor neuron disease (MND), but the molecular basis of these abnormalities is unknown. It is also unclear whether the observed mitochondrial dysfunction plays a central role in disease pathogenesis, and if so, whether its amelioration might present therapeutic opportunities. We adopted a candidate generation approach using proteomics to screen for changes in mitochondrial protein expression in a well-validated cell-culture model of superoxide dismutase 1 (SOD1) related familial MND (fMND). Changed proteins were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectroscopy. Protein candidates included apoptotic regulators, anti-oxidants and components of the electron transport chain. Confirmatory Western blotting was performed, and validated protein expression changes were further investigated. Peroxiredoxin 3 (Prx3), a mitochondrial thioredoxin-dependent hydroperoxidase, is downregulated in the presence of mutant SOD1 in both our cell-culture model and in the spinal cord mitochondria of mutant SOD1 transgenic mice. We confirm the expression of Prx3 within the mitochondria of spinal motor neurons in mouse and humans by immunohistochemistry. Using quantitative real-time PCR (Q-PCR), we show that Prx3 is also downregulated in spinal motor neurons from patients with both sporadic (sMND) and SOD1-related fMND. In a disease characterized by oxidative stress, this represents a potentially important deficit in mitochondrial anti-oxidant defence. Recent evidence suggests that oxidative stress from aberrant copper chemistry may not play a major part in the pathogenesis of SOD1-related fMND. From the results of this study we propose disruption of mitochondrial anti-oxidant defence as an alternative mechanism whereby mutant SOD1 may generate oxidative stress within motor neurons. We further demonstrate that ebselen, an anti-oxidant drug already safely used in human studies and that acts as a Prx mimic, is able to ameliorate the toxicity of mutant SOD1 in our cell-culture model. We conclude by showing that ebselen is capable of inducing transcription of the anti-oxidant response element (ARE) and postulate that ebselen may act both by the transcriptional upregulation of anti-oxidant proteins, and directly as an anti-oxidant in its own right.

3.772 Melanosomal sequestration of cytotoxic drugs contributes to the intractability of malignant melanomas
Chen, K.G. et al
*PNAS, **103(26)**, 9903-9907 (2006)*

Multidrug resistance mechanisms underlying the intractability of malignant melanomas remain largely unknown. In this study, we demonstrate that the development of multidrug resistance in melanomas

involves subcellular sequestration of intracellular cytotoxic drugs such as *cis*-diaminedichloroplatinum II (cisplatin; CDDP). CDDP is initially sequestered in subcellular organelles such as melanosomes, which significantly reduces its nuclear localization when compared with nonmelanoma/KB-3-1 epidermoid carcinoma cells. The melanosomal accumulation of CDDP remarkably modulates melanogenesis through a pronounced increase in tyrosinase activity. The altered melanogenesis manifested an ≈8-fold increase in both intracellular pigmentation and extracellular transport of melanosomes containing CDDP. Thus, our experiments provide evidence that melanosomes contribute to the refractory properties of melanoma cells by sequestering cytotoxic drugs and increasing melanosome-mediated drug export. Preventing melanosomal sequestration of cytotoxic drugs by inhibiting the functions of melanosomes may have great potential as an approach to improving the chemosensitivity of melanoma cells.

3.773 A Novel Mechanism of Interaction between α-Synuclein and Biological Membranes
Kim, Y.S., Laurine, E., Woods, W. and Lee, S-J.
J. Mol. Biol., ***360(2)***, *386-397 (2006)*

Conformational abnormalities and aggregation of α-synuclein (α-syn) have been linked to the pathogenesis of Parkinson's (PD) and related diseases. It has been shown that α-syn can stably bind artificial phospholipid vesicles through α-helix formation in its N-terminal repeat region. However, little is known about the membrane interaction in cells. In the current study, we determined the membrane-binding properties of α-syn to biological membranes by using bi-functional chemical crosslinkers, which allow the detection of transient, but specific, interactions. By utilizing various point mutations and deletions within α-syn, we demonstrated that the membrane interaction of α-syn in cells is also mediated by α-helix formation in the N-terminal repeat region. Moreover, the PD-linked A30P mutation causes reduced membrane binding, which is concordant with the artificial membrane studies. However, contrary to the interaction with artificial membranes, the interaction with biological membranes is rapidly reversible and is not driven by electrostatic attraction. Furthermore, the interaction of α-syn with cellular membranes occurs only in the presence of non-protein and non-lipid cytosolic components, which distinguishes it from the spontaneity of the interaction with artificial membranes. More interestingly, addition of the cytosolic preparation to artificial membranes resulted in the transient, charge-independent binding of α-syn similar to the interaction with biological membranes. These results suggest that in cells, α-syn is engaged in a fundamentally different mode of membrane interaction than the charge-dependent artificial membrane binding, and the mode of interaction is determined by the intrinsic properties of α-syn itself and by the cytoplasmic context.

3.774 Identification of Proteomic Signatures of Exposure to Marine Pollutants in Mussels (Mytilus edulis)
Apraiz, I., Mi, J. and Cristobal, S.
Mol. Cell. Proteomics, ***5(7)***, *1274-1285 (2006)*

Bivalves and especially mussels are very good indicators of marine and estuarine pollution, and so they have been widely used in biomonitoring programs all around the world. However, traditional single parameter biomarkers face the problem of high sensitivity to biotic and abiotic factors. In our study, digestive gland peroxisome-enriched fractions of *Mytilus edulis* (L., 1758) were analyzed by DIGE and MS. We identified several proteomic signatures associated with the exposure to several marine pollutants (diallyl phthalate, PBDE-47, and bisphenol-A). Animals collected from North Atlantic Sea were exposed to the contaminants independently under controlled laboratory conditions. One hundred and eleven spots showed a significant increase or decrease in protein abundance in the two-dimensional electrophoresis maps from the groups exposed to pollutants. We obtained a unique protein expression signature of exposure to each of those chemical compounds. Moreover a set of proteins composed a proteomic signature in common to the three independent exposures. It is remarkable that the principal component analysis of these spots showed a discernible separation between groups, and so did the hierarchical clustering into four classes. The 14 proteins identified by MS participate in α- and ß-oxidation pathways, xenobiotic and amino acid metabolism, cell signaling, oxyradical metabolism, peroxisomal assembly, respiration, and the cytoskeleton. Our results suggest that proteomic signatures could become a valuable tool to monitor the presence of pollutants in field experiments where a mixture of pollutants is often present. Further studies on the identified proteins could provide crucial information to understand possible mechanisms of toxicity of single xenobiotics or mixtures of them in marine ecosystems.

3.775 Outer Membrane Vesicle Production by Escherichia coli Is Independent of Membrane Instability
McBroom, A.J., Johnson, A.P., Vemulapalli, S. and Kuehn, M.J.
J. Bacteriol., ***188(15)***, *5385-5392 (2006)*

It has been long noted that gram-negative bacteria produce outer membrane vesicles, and recent data demonstrate that vesicles released by pathogenic strains can transmit virulence factors to host cells. However, the mechanism of vesicle release has remained undetermined. This genetic study addresses whether these structures are merely a result of membrane instability or are formed by a more directed process. To elucidate the regulatory mechanisms and physiological basis of vesiculation, we conducted a screen in *Escherichia coli* to identify gene disruptions that caused vesicle over- or underproduction. Only a few low-vesiculation mutants and no null mutants were recovered, suggesting that vesiculation may be a fundamental characteristic of gram-negative bacterial growth. Gene disruptions were identified that caused differences in vesicle production ranging from a 5-fold decrease to a 200-fold increase relative to wild-type levels. These disruptions included loci governing outer membrane components and peptidoglycan synthesis as well as the σ^E cell envelope stress response. Mutations causing vesicle overproduction did not result in upregulation of the *ompC* gene encoding a major outer membrane protein. Detergent sensitivity, leakiness, and growth characteristics of the novel vesiculation mutant strains did not correlate with vesiculation levels, demonstrating that vesicle production is not predictive of envelope instability.

3.776 Ribosomal Protein S6 Associates with Alphavirus Nonstructural Protein 2 and Mediates Expression from Alphavirus Messages

Montgomery, S.A., Berglund, P., Beard, C.W. and Johnston, R.E.
J. Virol., ***80(15)***, *7729-7739 (2006)*

Although alphaviruses dramatically alter cellular function within hours of infection, interactions between alphaviruses and specific host cellular proteins are poorly understood. Although the alphavirus nonstructural protein 2 (nsP2) is an essential component of the viral replication complex, it also has critical auxiliary functions that determine the outcome of infection in the host. To gain a better understanding of nsP2 function, we sought to identify cellular proteins with which Venezuelan equine encephalitis virus nsP2 interacted. We demonstrate here that nsP2 associates with ribosomal protein S6 (RpS6) and that nsP2 is present in the ribosome-containing fractions of a polysome gradient, suggesting that nsP2 associates with RpS6 in the context of the whole ribosome. This result was noteworthy, since viral replicase proteins have seldom been described in direct association with components of the ribosome. The association of RpS6 with nsP2 was detected throughout the course of infection, and neither the synthesis of the viral structural proteins nor the presence of the other nonstructural proteins was required for RpS6 interaction with nsP2. nsP1 also was associated with RpS6, but other nonstructural proteins were not. RpS6 phosphorylation was dramatically diminished within hours after infection with alphaviruses. Furthermore, a reduction in the level of RpS6 protein expression led to diminished expression from alphavirus subgenomic messages, whereas no dramatic diminution in cellular translation was observed. Taken together, these data suggest that alphaviruses alter the ribosome during infection and that this alteration may contribute to differential translation of host and viral messages.

3.777 Cytotoxic Necrotizing Factor Type 1 Delivered by Outer Membrane Vesicles of Uropathogenic Escherichia coli Attenuates Polymorphonuclear Leukocyte Antimicrobial Activity and Chemotaxis

Davis, J.M., Carvalho, H.M., Rasmussen, S.B. and O'Brien, A.D.
Infect. Immun., ***74(8)***, *4401-4408 (2006)*

Cytotoxic necrotizing factor type 1 (CNF1), a toxin produced by many strains of uropathogenic *Escherichia coli* (UPEC), constitutively activates small GTPases of the Rho family by deamidating a single amino acid within these target proteins. Such activated GTPases not only stimulate actin polymerization within affected cells but also, as we previously reported, decrease membrane fluidity on mouse polymorphonuclear leukocytes (PMNs). In that same investigation we found that this diminished membrane movement impedes the clustering of the complement receptor CD11b/CD18 on PMNs and, in turn, decreases PMN phagocytic capacity and microbicidal activity on PMNs in direct contact with CNF1-expressing UPEC as well as on those in proximity to wild-type UPEC. The latter observation suggested to us that CNF1 is released from neighboring bacteria, although at the time of initiation of the study described here, no specific mechanism for export of CNF1 from UPEC had been described. Here we present evidence that CNF1 is released from the CNF1-expressing UPEC strain CP9 (serotype O4/H5/K54) in a complex with outer membrane vesicles (OMVs) and that these CNF1-bearing vesicles transfer biologically active CNF1 to PMNs and attenuate phagocyte function. Furthermore, we show that CNF1-bearing vesicles act in a dose-dependent fashion on PMNs to inhibit their chemotactic response to formyl-Met-Leu-Phe, while purified CNF1 does not. We conclude that OMVs provide a means for delivery of CNF1 from a

UPEC strain to PMNs and thus negatively affect the efficacy of the acute inflammatory response to these organisms.

3.778 The Secretory Granule Protein Syncollin Localizes to HL-60 Cells and Neutrophils
Bach, J-P. et al
J. Histochem. Cytochem., ***54(8)***, *877-888 (2006)*

The secretory granule protein syncollin was first identified in the exocrine pancreas where a population of the protein is associated with the luminal surface of the zymogen granule membrane. In this study we provide first morphological and biochemical evidence that, in addition to its pancreatic localization, syncollin is also present in neutrophilic granulocytes of rat and human origin. By immunohistological studies, syncollin was detected in neutrophilic granulocytes of the spleen. Furthermore, syncollin is expressed by the promyelocytic HL-60 cells, where it is stored in azurophilic granules and in a vesicular compartment. These findings were confirmed by fractionation experiments and immunoelectron microscopy. Treatment with a phorbol ester triggered the release of syncollin indicating that in HL-60 cells it is a secretory protein that can be mobilized upon stimulation. A putative role for syncollin in host defense is discussed.

3.779 Cardiac aquaporin expression in humans, rats, and mice
Butler, T.L. et al
Am. J. Physiol. Heart Circ. Physiol., ***291***, *H705-H713 (2006)*

Water accumulation in the heart is important in ischemia-reperfusion injury and operations performed by using cardiopulmonary bypass, with cardiac dysfunction associated with myocardial edema being the principal determinant of clinical outcome. As an initial step in determining the role of aquaporin (AQP) water channels in myocardial edema, we have assessed the myocardial expression of AQPs in humans, rats, and mice. RT-PCR revealed expression of AQP-1, -4, -6, -7, -8, and -11 transcripts in the mouse heart. AQP-1, -6, -7, and -11 mRNAs were found in the rat heart as well as low levels of AQP-4 and -9. Human hearts contained AQP-1, -3, -4, -5, -7, -9, -10, and -11 mRNAs. AQP-1 protein expression was confirmed by Western blot analysis in all three species. AQP-4 protein was detected in the mouse heart but not in the rat or human heart. To determine the potential functional consequences of myocardial AQP expression, water permeability was measured in plasma membrane vesicles from myocardial cells of wild-type versus various AQP knockout mice. Water permeability was reduced by AQP-1 knockout but not by AQP-4 or AQP-8 knockout. With the use of a model of isolated rat heart perfusion, it was found that osmotic and ischemic stresses are not associated with changes in AQP-1 or AQP-4 expression. These studies support a possible functional role of AQP-1 in myocardium but indicate that early adaptations to osmotic and ischemic stress do not involve transcriptional or posttranslational AQP-1 regulation.

3.780 More than colocalizing with polycystin-1, polycystin-L is in the centrosome
Bui-Xuan, E-F. et al
Am. J. Physiol. Renal Physiol., ***291***, *F395-F406 (2006)*

Polycystin-1 and polycystin-2 are involved in autosomal dominant polycystic kidney disease by unknown mechanisms. These two proteins are located in primary cilia where they mediate mechanosensation, suggesting a link between cilia function and renal disease. In this study, we sought to characterize the subcellular localization of polycystin-L, a closely related member of polycystin-2, in epithelial renal cell lines. We have shown that endogenous polycystin-L subcellular distribution is different in proliferative and nonproliferative cultures. Polycystin-L is found mostly in the endoplasmic reticulum in subconfluent cell cultures, while in confluent cells it is redistributed to sites of cell-cell contact and to the primary cilium as is polycystin-1. Subcellular fractionation confirmed a common distribution of polycystin-L and polycystin-1 in the fractions corresponding to those containing the plasma membrane of postconfluent cells. Reciprocal coimmunoprecipitation experiments showed that polycystin-L was associated with polycystin-1 in a common complex in both subconfluent and confluent cell cultures. Interestingly, we also identified a novel site for a polycystin member (polycystin-L) in unciliated cells, the centrosome, which allowed us to reveal an involvement of polycystin-L in cell proliferation.

3.781 Association of Yeast Transporters with Detergent-Resistant Membranes Correlates with Their Cell-Surface Location
Lauwers, E. and Andre, B.
Traffic, ***7(8)***, *1045-1059 (2006)*

Detergent-resistant membrane (DRM) fractions enriched in ergosterol and sphingolipids can be isolated from yeast cells and have been proposed to represent the biochemical equivalents of lipid rafts. Most yeast plasma membrane proteins studied for their detergent solubility have been found in DRMs, except for the Hxt1 and Gap1 permeases. We here compared Gap1 detergent solubility in wild-type and various mutant cells under conditions promoting cell surface accumulation or ubiquitin-dependent down-regulation of the permease. We show that Gap1 present at the plasma membrane is associated with DRMs. This association occurs at the Golgi level. In the absence of sphingolipid neosynthesis, Gap1 fails to accumulate at the plasma membrane and is missorted to the vacuolar lumen. Furthermore, the presence of Gap1 at the plasma membrane correlates perfectly with its association with DRMs, whatever the activity or ubiquitination state of the permease and regardless of whether it has reached the cell surface via normal secretion, after recycling, or upon missorting to the vacuole before rerouting to the plasma membrane. Finally, we show that Hxt1 present at the cell surface is also associated with DRMs. We discuss a model where yeast plasma membrane proteins are systematically associated with sphingolipid/ergosterol-enriched microdomains when located at the cell surface.

3.782 Dynamic Sequestration of the Recycling Compartment by Classical Protein Kinase C
Idkowiak-Baldys, J., Becker, K.P., Kitatani, K. And Hannum, Y.A.
J. Biol. Chem., ***281(31)***, *22321-22331 (2006)*

It has been previously shown that upon sustained stimulation (30-60 min) with phorbol esters, protein kinase C (PKC)α and βII become sequestered in a juxtanuclear region, the pericentrion. The activation of PKC also results in sequestration of transferrin, suggesting a role for PKC in regulating endocytosis and sequestration of recycling components. In this work we characterize the pericentrion as a PKC-dependent subset of the recycling compartment. We demonstrate that upon sustained stimulation of PKC, both protein (CD59, caveolin) and possibly also lipid (Bodipy-GM1) cargo become sequestered in a PKC-dependent manner. This sequestration displayed a strict temperature requirement and was inhibited below 32 °C. Treatment of cells with phorbol myristate acetate for 60 min led to the formation of a distinct membrane structure. PKC sequestration and pericentrion formation were blocked by hypertonic sucrose as well as by potassium depletion (inhibitors of clathrin-dependent endocytosis) but not by nystatin or filipin, which inhibit clathrin-independent pathways. Interestingly, it was also observed that some molecules that internalize through clathrin-independent pathways (CD59, Bodipy-GM1, caveolin) also sequestered to the pericentrion upon sustained PKC activation, suggesting that PKC acted distal to the site of internalization of endocytic cargo. Together these results suggest that PKC regulates sequestration of recycling molecules into this compartment, the pericentrion.

3.783 Pathogenic mutations of presenilins enhance pro-apoptotic activity by reducing mitochondrial Bcl-2
Wang, H-Q. et al
Alzheimer's and Dementia, ***2(3)***, *Supplement 1, Page S494 (2006)*

Background: The mechanism of neuronal loss in Alzheimer's disease (AD) remains unknown, although circumstantial evidence of apoptosis in postmortem brain tissues has supported the hypothesis that an apoptotic mechanism plays a role in the neurodegeneration in AD. Mutations in presenilins 1 and 2 (PS1/2) are responsible for the majority of familial AD. These proteins are localized predominantly in the endoplasmic reticulum (ER) and Golgi apparatus, and form heteromeric protein complexes to participate in several functions. Previous studies have shown that PS1/2 are involved in the regulation of apoptotic cell death, although the molecular basis is still unresolved. **Objective(s):** The aim of this study is to clarify the molecular basis by which PS1/2 regulate apoptosis. We also address a question how presenilin mutations modify the regulatory activity. **Methods:** We performed yeast 2-hybrid screen, co-immunoprecipitation assay, glycerol velocity gradient centrifugation and subcellular fractionation with iodixanol gradient, using HEK293 cells, PS1/2-knockout mouse fibroblasts and I213T-PS1-knockin mouse brains. **Results:** PS1/2 interacted with FKBP38 that is an immunophilin family member residing in the mitochondrial membrane and inhibits apoptosis by targeting and anchoring antiapoptotic Bcl-2 to the mitochondria. PS1/2 and FKBP38 formed macromolecular complexes together with Bcl-2. This complex formation promoted the degradation of FKBP38 and Bcl-2, and sequestered these proteins in the ER/Golgi compartments, thereby inhibiting FKBP38-mediated mitochondrial targeting of Bcl-2. Thus, full-length PS1/2 increased the susceptibility to apoptosis by antagonizing the anti-apoptotic function of FKBP38. In contrast, C-terminal fragments of caspase-processed PS1/2 redistributed Bcl-2 to the mitochondria by abrogating the activity of full-length PS1/2, resulting in a dominant-negative anti-

apoptotic effect. In cultured cells and mutant PS1-knockin mouse brains, familial AD-linked PS1/2 mutants enhanced the pro-apoptotic activity by causing a more efficient reduction in mitochondrial Bcl-2 than wild-type PS1/2. **Conclusions:** We demonstrated that PS1/2 regulate the susceptibility to apoptosis by interacting with FKBP38. These results suggest a novel molecular mechanism for the regulation of mitochondria-mediated apoptosis by competition between PS1/2 and FKBP38 for subcellular targeting of Bcl-2. Furthermore, familial AD-linked mutations in PS1/2 could lower the threshold for activation of mitochondria-mediated apoptosis pathways inneurons of individuals with familial AD.

3.784 Accumulation of sphingolipids increases secretion of the amyloid β-peptide by stabilization of the β-amyloid precursor protein

Hampel, H., Sandhoff, K. and Walter, J.
Alzheimer's and Dementia, 2(3), Supplement 1, Pages S528-S529 (2006)

Alzheimer's disease is associated with extracellular deposits in the brain of amyloid-_-peptides (A_) that are generated by proteolytic processing of the _-amyloid precursor protein (APP). It has been shown that membrane lipids, including cholesterol and sphingolipids affect the subcellular transport of APP in the secretory pathway and its proteolytic processing. Previously, we also showed that the inhibition of glycosphingolipid (GSL) biosynthesis reduces the secretion of APP and A_. Sphingolipids are highly enriched in the plasma membrane of cells. From here they are transported to late endosomal/lysosomal compartments where they are degraded. Inherited defects in degradation of these lipids cause sphingolipid storage disorders (SLSDs) marked by accumulation of GSLs in neurons associated with severe neurodegeneration. There is also a common defect in lipid transport along the endocytic pathway in SLSDs. Since processing of APP by secretases also occurs predominantly in post- Golgi secretory and endocytic compartments, we investigated the effect of accumulation of sphingolipids on transport and processing of APP. We used cultured cells and experimentally increased GSL levels by incubation with bovine brain gangliosides. The proteolytic processing of APP and its derivatives was studied by the detection of full length and soluble APP, APP C-terminal fragments (CTFs) as well as A_ and by pulse-chase experiments. Distribution of APP and its processing products was analyzed by iodixanol density gradient. The accumulation of GSLs markedly increased the secretion of endogenous APP and A_. A strong increase in total APP-CTF levels upon addition of GSLs was also observed. Addition of sphingomyelin showed similar effects. However _-secretase activity was not affected by GSLs in in vitro assays. By biochemical and cell biological experiments, we demonstrate that the increased levels of cellular GSLs altered the distribution and stability of APP-CTFs. Similar results were also obtained in independent genetic cellular models of GSL storage. On the other hand, GSL deficient cells showed decreased levels of APP-CTFs. Together, these data demonstrate that GSLs enhance the secretion of A_ likely by stabilizing APP-CTFs, thereby providing more substrate for _-secretases. Our studies suggest a novel role of GSLs in regulation of APP trafficking and A_ generation along the endocytic pathway.

3.785 YKE4 (YIL023C) Encodes a Bidirectional Zinc Transporter in the Endoplasmic Reticulum of Saccharomyces cerevisiae

Kumanovics, A., Poruk, K.E., Osborn, K.A., Ward, D.M. and Kaplan, J.
*J. Biol. Chem., **281(32)**, 22566-22574 (2006)*

YIL023C encodes a member of the SLC39A, or ZIP, family, which we refer to as yeast KE4 (*YKE4*) after its mouse ortholog. Yke4p was localized to the endoplasmic reticulum (ER) membrane using Yke4p-specific antiserum. *YKE4* is not an essential gene; however, deletion of *YKE4* resulted in a sensitivity to calcofluor white and poor growth at 36 °C on respiratory substrates containing high zinc. Overexpression of transition metal transporters Zrc1p and Cot1p or the mouse orthologue mKe4 in Δ*yke4* suppressed the poor growth at 36 °C on respiratory substrates. We found that the role of Yke4p depends on the zinc status of the cells. In a zinc-adequate environment, Yke4p transports zinc into the secretory pathway, and the deletion of *YKE4* leads to a zinc-suppressible cell wall defect. In high zinc medium, transport of zinc into the secretory pathway through Yke4p is a way to eliminate zinc from the cytosol, and deletion of *YKE4* leads to toxic zinc accumulation in the cytosol. Under low cytosolic zinc conditions, however, Yke4p removes zinc from the secretory pathway, and deletion of *YKE4* partially compensates for the loss of Msc2p, an ER zinc importer, and therefore helps to alleviate ER stress. In our model, Yke4p balances zinc levels between the cytosol and the secretory pathway, whereas the previously described Msc2p-Zrg17p ER zinc importer complex functions mainly in zinc-depleted conditions to ensure a ready supply of zinc essential for ER functions, such as phospholipid biosynthesis and unfolded protein response.

3.786 Release of iron from ferritin requires lysosomal activity
Kidane, T.Z., Sauble, E. and Linder, M.C.
Am. J. Physiol. Cell Physiol., ***291***, *C445-C455 (2006)*

How ferritin-Fe becomes available for cell functions is unknown. Our previous studies with rat hepatoma cells indicated ferritin had to be degraded to release its Fe. In these studies, we investigated whether this occurs in other cell types and whether lysosomes are required. Release of ferritin-Fe was induced with desferoxamine (DFO) in ^{59}Fe-preloaded hepatoma, Caco2, and erythroid K562 cells and measured by rocket immunoelectrophoresis and autoradiography. The half-lives for ferritin-^{59}Fe and protein were parallel (23, 16, and 11 h for the hepatic, Caco2, and K562 cells, respectively). Co-treatment with 180 μM Fe, leupeptin, chymostatin, or chloroquine markedly decreased rates of ferritin-Fe release and ferritin degradation. Lactacystin had no effect except for a small one in erythroid cells. Fractionation of hepatoma cell lysates on **iodixanol** gradients showed rapid depletion of cytosolic ferritin by DFO treatment but no accumulation in lysosomes. We conclude that regardless of cell type, release of Fe from ferritin occurs mainly through lysosomal proteolysis.

3.787 Lipid rafts mediate ultraviolet light-induced Fas aggregation in M624 melanoma cells
Elyassaki, W. and Wu, S.
Photochem. Photobiol., ***82***, *787-792 (2006)*

Ultraviolet light (UV) induces aggregation of Fas-receptor through a Fas-ligand-independent pathway. However, the mechanism of ultraviolet light-induced Fas-receptor aggregation is not known. In this report, we show that lipid rafts mediate ultraviolet light-induced aggregation of Fas. Our data show that UV induces a redistribution of Fas-receptor in a 25-5% Optiprep continuous gradient. The amount of Fas-receptorS is significantly increased in a gradient fraction that contain lipid rafts and is associated with an increase of FADD and caspase-8. Our data also show that the active dimeric form of caspase-8 (p44/p41) is increased in the lipid raft fraction. In addition, our data show that cholesterol, a major component of lipid rafts, is significantly reduced in only the lipid raft fractions after UV-irradiation. However, ceramide, another major lipid raft component, is increased evenly in all gradient fractions after UV-irradiation. These results suggest that UV alters the composition of major lipid raft components, which leads to the recruitment of Fas-receptor and FADD, with subsequent activation of caspase-8. Based on our results, we propose a novel mechanism by which UV induces apoptosis through a membrane lipid raft-mediated signaling pathway.

3.788 Mitchell Medical Lecture: OH radical formation from the lysosomal electron carriers
Yoshida, M. et al
Biochim. Biophys. Acta, ***1757 (5-6), Suppl. 1,*** *217 (2006)*

No abstract available

3.789 Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments
Lampugnani, M.G., Orsenigo, F., Gagliani, M.C., Tacchetti, C. and Dejana, E.
J. Cell Biol., ***174(4)***, *593-604 (2006)*

Receptor endocytosis is a fundamental step in controlling the magnitude, duration, and nature of cell signaling events. Confluent endothelial cells are contact inhibited in their growth and respond poorly to the proliferative signals of vascular endothelial growth factor (VEGF). In a previous study, we found that the association of vascular endothelial cadherin (VEC) with VEGF receptor (VEGFR) type 2 contributes to density-dependent growth inhibition (Lampugnani, G.M., A. Zanetti, M. Corada, T. Takahashi, G. Balconi, F. Breviario, F. Orsenigo, A. Cattelino, R. Kemler, T.O. Daniel, and E. Dejana. 2003. *J. Cell Biol.* 161:793–804). In the present study, we describe the mechanism through which VEC reduces VEGFR-2 signaling. We found that VEGF induces the clathrin-dependent internalization of VEGFR-2. When VEC is absent or not engaged at junctions, VEGFR-2 is internalized more rapidly and remains in endosomal compartments for a longer time. Internalization does not terminate its signaling; instead, the internalized receptor is phosphorylated, codistributes with active phospholipase C-γ, and activates p44/42 mitogen-activated protein kinase phosphorylation and cell proliferation. Inhibition of VEGFR-2 internalization reestablishes the contact inhibition of cell growth, whereas silencing the junction-associated density-enhanced phosphatase-1/CD148 phosphatase restores VEGFR-2 internalization and signaling. Thus, VEC

limits cell proliferation by retaining VEGFR-2 at the membrane and preventing its internalization into signaling compartments.

3.790 The membrane proximal disulfides of the EGF receptor extracellular domain are required for high affinity binding and signal transduction but do not play a role in the localization of the receptor to lipid rafts
Macdonald, J., Li, Z., Su, W. and Pike, L.J.
*Biochim. Biophys. Acta, **1763(8)**, 870-878 (2006)*

The EGF receptor is a transmembrane receptor tyrosine kinase that is enriched in lipid rafts. Subdomains I, II and III of the extracellular domain of the EGF receptor participate in ligand binding and dimer formation. However, the function of the cysteine-rich subdomain IV has not been elucidated. In this study, we analyzed the role of the membrane-proximal portion of subdomain IV in EGF binding and signal transduction. A double Cys → Ala mutation that breaks the most membrane-proximal disulfide bond (Cys600 to Cys612), ablated high affinity ligand binding and substantially reduced signal transduction. A similar mutation that breaks the overlapping Cys596 to Cys604 disulfide had little effect on receptor function. Mutation of residues within the Cys600 to Cys612 disulfide loop did not alter the ligand binding or signal transducing activities of the receptor. Despite the fact that the C600,612A EGF receptor was significantly impaired functionally, this receptor as well as all of the other receptors with mutations in the region of residues 596 to 612 localized normally to lipid rafts. These data suggest that the disulfide-bonded structure of the membrane-proximal portion of the EGF receptor, rather than its primary sequence, is important for EGF binding and signaling but is not involved in localizing the receptor to lipid rafts.

3.791 RGS Expression Rate-Limits Recovery of Rod Photoresponses
Krispel, C.M. et al
*Neurons, **51(4)**, 409-416 (2006)*

Signaling through G protein-coupled receptors (GPCRs) underlies many cellular processes, yet it is not known which molecules determine the duration of signaling in intact cells. Two candidates are G protein-coupled receptor kinases (GRKs) and Regulators of G protein signaling (RGSs), deactivation enzymes for GPCRs and G proteins, respectively. Here we investigate whether GRK or RGS governs the overall rate of recovery of the light response in mammalian rod photoreceptors, a model system for studying GPCR signaling. We show that overexpression of rhodopsin kinase (GRK1) increases phosphorylation of the GPCR rhodopsin but has no effect on photoresponse recovery. In contrast, overexpression of the photoreceptor RGS complex (RGS9-1·Gβ5L·R9AP) dramatically accelerates response recovery. Our results show that G protein deactivation is normally at least 2.5 times slower than rhodopsin deactivation, resolving a long-standing controversy concerning the mechanism underlying the recovery of rod visual transduction.

3.792 The Use of GFP to Localize Rho GTPases in Living Cells
Michaelson, D. and Philips, M.
*Methods in Enzymol., **406**, 296-315 (2006)*

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has revolutionized the study of protein localization and dynamics. GFP fusions permit analysis of proteins in living cells and offer distinct advantages over conventional immunofluorescence. Among these are lower background, higher resolution, robust dual color colocalization, and avoidance of fixation artifacts. In the case of Ras and Rho family proteins, GFP fusions have allowed breakthroughs in the understanding of how CAAX proteins are targeted to specific cell membranes and how signaling at different membranes can result in different cellular responses. GFP-tagged Rho proteins have also been informative in analyzing the interactions with the cytosolic chaperone, RhoGDI. The major disadvantages of studying GFP fusion proteins is that they are generally overexpressed relative to endogenous proteins, and the GFP tag can, in principle, affect protein function. Fortunately, in the case of Ras and Rho family proteins, a GFP tag at the N terminus seems to have little effect on protein targeting and function. Nevertheless, it is prudent to confirm GFP fusion protein data with the study of the endogenous protein. This chapter describes the tagging of Rho proteins with GFP and the analysis of GFP–Rho protein localization by epifluorescence and confocal microscopy. It further describes methods of analyzing endogenous Rho proteins as confirmation of data acquired using GFP–Rho fusion proteins. These techniques will be useful for anyone studying Rho protein function and are widely applicable to many cell types and signal transduction systems.

3.793 Activation process of the mosquitocidal δ-endotoxin Cry39A produced by Bacillus thuringiensis subsp. aizawai BUN1-14 and binding property to Anopheles stephensi BBMV
Ito, T., Bando, H. and Asano, S-i.
J. Invertebrate Pathol., ***93(1)***, *29-35 (2006)*

Most δ-endotoxins produced by *Bacillus thuringiensis* require proteolytic processing in order to become active. The *in vitro* and *in vivo* activation processes of Cry39A, a δ-endotoxin that is highly toxic to *Anopheles stephensi*, were investigated. Cry39A with a molecular mass of 72 kDa was processed *in vitro* into a 60 kDa fragment by trypsin and gut extract from *A. stephensi* larvae. N-terminal amino acid sequencing of the 60 kDa fragment revealed that trypsin and the protease(s) in the gut extract cleaved Cry39A between Arg^{61} and Gly^{62}. In contrast, 40 and 25 kDa polypeptides were generated *in vivo* by intramolecular cleavage of the 60 kDa fragment in *A. stephensi* larvae. Further, a co-precipitation assay was used to investigate the binding property of the activated Cry39A to *A. stephensi* BBMV. Cry39A bound to *A. stephensi* BBMV specifically and did not compete with the Cry4Aa toxin. This indicated that the binding molecule(s) for Cry39A might differ from those for Cry4A. In addition, Cry39A preferentially bound to the Triton X-100-insoluble membrane fraction.

3.794 Endogenous spartin, mutated in hereditary spastic paraplegia, has a complex subcellular localization suggesting diverse roles in neurons
Robay, D., Patel, H., Simpson, M.A., Brown, N.A. and Crosby, A.H.
Exp. Cell Res., ***312(15)***, *2764-2777 (2006)*

Mutation of spartin (SPG20) underlies a complicated form of hereditary spastic paraplegia, a disorder principally defined by the degeneration of upper motor neurons. Using a polyclonal antibody against spartin to gain insight into the function of the endogenous molecule, we show that the endogenous molecule is present in two main isoforms of 85 kDa and 100 kDa, and 75 kDa and 85 kDa in human and murine, respectively, with restricted subcellular localization. Immunohistochemical studies on human and mouse embryo sections and in vitro cell studies indicate that spartin is likely to possess both nuclear and cytoplasmic functions. The nuclear expression of spartin closely mirrors that of the snRNP (small nuclear ribonucleoprotein) marker α-Sm, a component of the spliceosome. Spartin is also enriched at the centrosome within mitotic structures. Notably we show that spartin protein undergoes dynamic positional changes in differentiating human SH-SY5Y cells. In undifferentiated non-neuronal cells, spartin displays a nuclear and diffuse cytosolic profile, whereas spartin transiently accumulates in the *trans*-Golgi network and subsequently decorates discrete puncta along neurites in terminally differentiated neuroblastic cells. Investigation of these spartin-positive vesicles reveals that a large proportion colocalizes with the synaptic vesicle marker synaptotagmin. Spartin is also enriched in synaptic-like structures and in synaptic vesicle-enriched fraction.

3.795 Cholesterol depletion reduces aggregation of amyloid-beta peptide in hippocampal neurons
Scheider, A., Schulz-Schaeffer, W., Hartmann, T., Schulz, J.B. and Simons, M.
Neurobiology of Disease, ***23***, *573-577 (2006)*

A key event in the pathogenesis of Alzheimer's disease is the conversion of soluble amyloid Aβ-peptide into toxic aggregates. Here, we studied the effect of cholesterol depletion on the formation of insoluble Aβ. We found that reduction of neuronal cholesterol by ~25% reduced the neuronal formation of insoluble Aβ without affecting the secretion of soluble Aβ. Moreover, we demonstrate that Aβ-oligomers from Alzheimer's disease brains associate with a detergent-resistant membrane fraction in a cholesterol-dependent manner. These results suggest a key role for cholesterol in aggregation of Aβ.

3.796 Cotranslocational Degradation Protects the Stressed Endoplasmic Reticulum from Protein Overload
Oyadomari, S. et al
Cell, ***126(4)***, *727-739 (2006)*

The ER's capacity to process proteins is limited, and stress caused by accumulation of unfolded and misfolded proteins (ER stress) contributes to human disease. ER stress elicits the unfolded protein response (UPR), whose components attenuate protein synthesis, increase folding capacity, and enhance misfolded protein degradation. Here, we report that $P58^{IPK}$/DNAJC3, a UPR-responsive gene previously implicated in translational control, encodes a cytosolic cochaperone that associates with the ER protein translocation channel Sec61. $P58^{IPK}$ recruits HSP70 chaperones to the cytosolic face of Sec61 and can be crosslinked to

proteins entering the ER that are delayed at the translocon. Proteasome-mediated cytosolic degradation of translocating proteins delayed at Sec61 is cochaperone dependent. In $P58^{IPK-/-}$ mice, cells with a high secretory burden are markedly compromised in their ability to cope with ER stress. Thus, $P58^{IPK}$ is a key mediator of cotranslocational ER protein degradation, and this process likely contributes to ER homeostasis in stressed cells.

3.797 Translocation of Endothelial Nitric-Oxide Synthase Involves a Ternary Complex with Caveolin-1 and NOSTRIN
Schilling, K. et al
*Mol. Biol. Cell, **17**, 3870-3880 (2006)*

Recently, we characterized a novel endothelial nitric-oxide synthase (eNOS)-interacting protein, NOSTRIN (for e*NOS-t*rafficking *in*ducer), which decreases eNOS activity upon overexpression and induces translocation of eNOS away from the plasma membrane. Here, we show that NOSTRIN directly binds to caveolin-1, a well-established inhibitor of eNOS. Because this interaction occurs between the N terminus of caveolin (positions 1–61) and the central domain of NOSTRIN (positions 323–434), it allows for independent binding of each of the two proteins to eNOS. Consistently, we were able to demonstrate the existence of a ternary complex of NOSTRIN, eNOS, and caveolin-1 in Chinese hamster ovary (CHO)-eNOS cells. In human umbilical vein endothelial cells (HUVECs), the ternary complex assembles at the plasma membrane upon confluence or thrombin stimulation. In CHO-eNOS cells, NOSTRIN-mediated translocation of eNOS involves caveolin in a process most likely representing caveolar trafficking. Accordingly, trafficking of NOSTRIN/eNOS/caveolin is affected by altering the state of actin filaments or cholesterol levels in the plasma membrane. During caveolar trafficking, NOSTRIN functions as an adaptor to recruit mediators such as dynamin-2 essential for membrane fission. We propose that a ternary complex between NOSTRIN, caveolin-1, and eNOS mediates translocation of eNOS, with important implications for the activity and availability of eNOS in the cell.

3.798 An Internal EELD Domain Facilitates Mitochondrial Targeting of Mcl-1 via a Tom70-dependent Pathway
Chou, C-H., Lee, R-S., Yang-Yen, H-F.
*Mol. Biol. Cell, **17**, 3952-3963 (2006)*

Mcl-1 functions at an apical step in many regulatory programs that control cell death. Although the mitochondrion is one major subcellular organelle where Mcl-1 functions, the molecular mechanism by which Mcl-1 is targeted to mitochondria remains unclear. Here, we demonstrate that Mcl-1 is loosely associated with the outer membrane of mitochondria. Furthermore, we demonstrate that Mcl-1 interacts with the mitochondrial import receptor Tom70, and such interaction requires an internal domain of Mcl-1 that contains an EELD motif. A Tom70 antibody that blocks Mcl-1–Tom70 interaction blocks mitochondrial import of Mcl-1 in vitro. Furthermore, Mcl-1 is significantly less targeted to mitochondria in Tom70 knockdown than in the control cells. Similar targeting preference is also observed for the DM mutant of Mcl-1 whose mutation at the EELD motif markedly attenuates its Tom70 binding activity. Together, our results indicate that the internal EELD domain facilitates mitochondrial targeting of Mcl-1 via a Tom70-dependent pathway.

3.799 Cumulative Mutations Affecting Sterol Biosynthesis in the Yeast Saccharomyces cerevisiae Result in Synthetic Lethality That Is Suppressed by Alterations in Sphingolipid Profiles
Valachovic, M. et al
*Genetics, **173**, 1893-1908 (2006)*

UPC2 and *ECM22* belong to a Zn(2)–Cys(6) family of fungal transcription factors and have been implicated in the regulation of sterol synthesis in *Saccharomyces cerevisiae* and *Candida albicans*. Previous reports suggest that double deletion of these genes in *S. cerevisiae* is lethal depending on the genetic background of the strain. In this investigation we demonstrate that lethality of *upc2*Δ *ecm22*Δ in the S288c genetic background is attributable to a mutation in the *HAP1* transcription factor. In addition we demonstrate that strains containing *upc2*Δ *ecm22*Δ are also inviable when carrying deletions of *ERG6* and *ERG28* but not when carrying deletions of *ERG3*, *ERG4*, or *ERG5*. It has previously been demonstrated that *UPC2* and *ECM22* regulate *S. cerevisiae ERG2* and *ERG3* and that the *erg2*Δ *upc2*Δ *ecm22*Δ triple mutant is also synthetically lethal. We used transposon mutagenesis to isolate viable suppressors of *hap1*Δ, *erg2*Δ, *erg6*Δ, and *erg28*Δ in the *upc2*Δ *ecm22*Δ genetic background. Mutations in two genes (*YND1* and *GDA1*) encoding apyrases were found to suppress the synthetic lethality of three of these triple mutants

but not *erg2*Δ *upc2*Δ *ecm22*Δ. We show that deletion of *YND1*, like deletion of *GDA1*, alters the sphingolipid profiles, suggesting that changes in sphingolipids compensate for lethality produced by changes in sterol composition and abundance.

3.800 Membrane Association of the Cycling Peroxisome Import Receptor Pex5p
Kerssen, D. et al
J. Biol. Chem., ***281(37)***, *27003-27015 (2006)*

Peroxisomal proteins carrying a peroxisome targeting signal type 1 (PTS1) are recognized in the cytosol by the cycling import receptor Pex5p. The receptor-cargo complex docks at the peroxisomal membrane where it associates with multimeric protein complexes, referred to as the docking and RING finger complexes. Here we have identified regions within the *Saccharomyces cerevisiae* Pex5p sequence that interconnect the receptor-cargo complex with the docking complex. Site-directed mutagenesis of the conserved tryptophan residue within a reverse W*XXX*F motif abolished two-hybrid binding with the N-terminal half of Pex14p. In combination with an additional mutation introduced into the Pex13p-binding site, we generated a Pex5p mutant defective in a stable association not only with the docking complex but also with the RING finger peroxins at the membrane. Surprisingly, PTS1 proteins are still imported into peroxisomes in these mutant cells. Because these mutations had no significant effect on the membrane binding properties of Pex5p, we examined yeast and human Pex5p for intrinsic lipid binding activity. *In vitro* analyses demonstrated that both proteins have the potential to insert spontaneously into phospholipid membranes. Altogether, these data strongly suggest that a translocation-competent state of the PTS1 receptor enters the membrane via protein-lipid interactions before it tightly associates with other peroxins.

3.801 Palmitoylation Plays a Role in Targeting Vac8p to Specific Membrane Subdomains
Peng, Y., Tang, F. and Weisman, L.S.
Traffic, ***7***, *1378-1387 (2006)*

Vac8p is a multifunctional yeast protein involved in several distinct vacuolar events including vacuole inheritance, vacuole homotypic fusion, nucleus–vacuole junction formation and the cytoplasm to vacuole protein targeting pathway. Vac8p associates with the vacuole membrane via myristoylation and palmitoylation. Vac8p has three putative palmitoylation sites, at Cys 4, 5 and 7. Here, we show that each of these cysteines may serve as a palmitoylation site. Palmitoylation at Cys 7 alone provides partial function of Vac8p, whereas palmitoylation at either Cys 4 or Cys 5 alone is sufficient for Vac8p function. In the former mutant, there is a severe defect in the localization of Vac8p to the vacuole membrane, while in the latter mutants, there is a partial defect in the localization of Vac8p. In addition, our studies provide evidence that palmitoylation targets Vac8p to specific membrane subdomains.

3.802 Invasion of Host Cells by JC Virus Identifies a Novel Role for Caveolae in Endosomal Sorting of Noncaveolar Ligands
Querbes, W., O'Hara, B.A., Williams, G. and Arwood, W.J.
J. Virol., ***80(19)***, *9402-9413 (2006)*

Invasion of glial cells by the human polyomavirus, JC virus (JCV), leads to a rapidly progressing and uniformly fatal demyelinating disease known as progressive multifocal leukoencephalopathy. The endocytic trafficking steps used by JCV to invade cells and initiate infection are not known. We demonstrated that JCV infection was inhibited by dominant defective and constitutively active Rab5-GTPase mutants that acted at distinct steps in endosomal sorting. We also found that labeled JCV colocalized with labeled cholera toxin B and with caveolin-1 (cav-1) on early endosomes following internalization by clathrin-dependent endocytosis. JCV entry and infection were both inhibited by dominant defective mutants of eps15 and Rab5-GTPase. Expression of a dominant-negative scaffolding mutant of cav-1 did not inhibit entry or infection by JCV. A single-cell knockdown experiment using cav-1 shRNA did not inhibit JCV entry but interfered with a downstream trafficking event important for infection. These data show that JCV enters cells by clathrin-dependent endocytosis, is transported immediately to early endosomes, and is then sorted to a caveolin-1-positive endosomal compartment. This latter step is dependent on Rab5-GTPase, cholesterol, caveolin-1, and pH. This is the first example of a ligand that enters cells by clathrin-dependent endocytosis and is then sorted from early endosomes to caveosomes, indicating that caveolae-derived vesicles play a more important role than previously realized in sorting cargo from early endosomes.

3.803 Reconstitution of Herpes Simplex Virus Type 1 Nuclear Capsid Egress In Vitro
Remillard-Labrosse, G., Guay, G. and Lippe, R.
J. Virol., ***80(19)***, *9741-9753 (2006)*

Newly assembled herpesvirus capsids travel from the nucleus to the plasma membrane by a mechanism that is poorly understood. Furthermore, the contribution of cellular proteins to this egress has yet to be clarified. To address these issues, an in vitro nuclear egress assay that reproduces the exit of herpes simplex virus type 1 (HSV-1) capsids from nuclei isolated from infected cells was established. As expected, the assay has all the hallmarks of intracellular transport assays, namely, a dependence on time, energy, and temperature. Surprisingly, it is also dependent on cytosol and was slightly enhanced by infected cytosol, suggesting an implication of both host and viral proteins in the process. The capsids escaped these nuclei by budding through the inner nuclear membrane, accumulated as enveloped capsids between the two nuclear membranes, and were released in cytosol exclusively as naked capsids, exactly as in intact cells. This is most consistent with the view that the virus escapes by crossing the two nuclear membranes rather than through nuclear pores. Unexpectedly, nuclei isolated at the nonpermissive temperature from cells infected with a U_L26 thermosensitive protease mutant (V701) supported capsid egress. Although electron microscopy, biochemical, and PCR analyses hinted at a likely reconstitution of capsid maturation, DNA encapsidation could not be confirmed by a traditional SQ test. This assay should prove very useful for identification of the molecular players involved in HSV-1 nuclear egress.

3.804 Characterization of erasin (UBXD2): a new ER protein that promotes ER-associated protein degradation
Liang, J. et al
J. Cell Sci., ***119***, *4011-4024 (2006)*

Ubiquitin regulator-X (UBX) is a discrete protein domain that binds p97/valosin-containing protein (VCP), a molecular chaperone involved in diverse cell processes, including endoplasmic-reticulum-associated protein degradation (ERAD). Here we characterize a human UBX-containing protein, UBXD2, that is highly conserved in mammals, which we have renamed erasin. Biochemical fractionation, immunofluorescence and electron microscopy, and protease protection experiments suggest that erasin is an integral membrane protein of the endoplasmic reticulum and nuclear envelope with both its N- and C-termini facing the cytoplasm or nucleoplasm. Localization of GFP-tagged deletion derivatives of erasin in HeLa cells revealed that a single 21-amino-acid sequence located near the C-terminus is necessary and sufficient for localization of erasin to the endoplasmic reticulum. Immunoprecipitation and GST-pulldown experiments confirmed that erasin binds p97/VCP via its UBX domain. Additional immunoprecipitation assays indicated that erasin exists in a complex with other p97/VCP-associated factors involved in ERAD. Overexpression of erasin enhanced the degradation of the ERAD substrate CD3δ, whereas siRNA-mediated reduction of erasin expression almost completely blocked ERAD. Erasin protein levels were increased by endoplasmic reticulum stress. Immunohistochemical staining of brain tissue from patients with Alzheimer's disease and control subjects revealed that erasin accumulates preferentially in neurons undergoing neurofibrillary degeneration in Alzheimer's disease. These results suggest that erasin may be involved in ERAD and in Alzheimer's disease.

3.805 Characterization of Proline-Serine-Rich Carboxyl Terminus in Human Sulfotransferase 2B1b: Immunogenicity, Subcellular Localization, Kinetic Properties, and Phosphorylation
He, D. and Falany, C.N.
Drug. Metab. Dispos., ***34(10)***, *1749-1755 (2006)*

The human sulfotransferase (*SULT*) *2B1* gene is a member of the *SULT2* gene family and encodes two isoforms, SULT2B1a and SULT2B1b. Although messages for both *SULT2B1a* and SULT2B1b are detectable in human tissues, only SULT2B1b has been identified immunologically. Compared with other human SULTs, SULT2B1b has an extension at the proline- and serine-rich carboxyl (PSC) end of about 53 amino acids. The structure and function of this unique PSC extension were investigated. Constructs of full-length SULT2B1b as well as truncated SULT2B1b without the PSC extension were expressed in *Escherichia coli*. Removal of the PSC extension significantly decreased the thermostability of the expressed enzyme as well as decreasing the rate of dehydroepiandrosterone sulfation. Rabbit polyclonal antibodies were raised against both the full-length and truncated SULT2B1b proteins. Immunoblot analysis showed that antibodies raised to full-length SULT2B1b immunoreact only with full-length SULT2B1b, whereas antibodies raised to truncated SULT2B1b react with both full-length and truncated SULT2B1b. Unlike full-length SULT2B1b, truncated SULT2B1b was incapable of translocation to nuclei in transfected

human BeWo choriocarcinoma cells. Phosphorylated serines were detected in the PSC extension of full-length SULT2B1b expressed in BeWo cells but not in truncated SULT2B1b. At least one phosphorylated serine was detected in expressed SULT2B1b via two-dimensional gel electrophoresis, immunoblot analysis, and mass spectroscopic analysis. Bacterially expressed full-length SULT2B1b but not truncated SULT2B1b was phosphorylated by casein kinase or Cdc2 protein kinase in vitro. This study suggests that the PSC extension of SULT2B1b is an important site in the immunogenicity, nuclear translocation, kinetic activity, and thermostability of this SULT isoform.

3.806 The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Human Breast Cancer Cell Proliferation through a Lipid Raft-Mediated Mechanism
Sarnataro, D. Et al
*Mol. Pharmacol., **70(4)**, 1298-1306 (2006)*

The endocannabinoid system has been shown to modulate key cell-signaling pathways involved in cancer cell growth. In this study, we show that cannabinoid receptor type 1 (CB1) antagonist Rimonabant (SR141716) inhibited human breast cancer cell proliferation, being more effective in highly invasive metastatic MDA-MB-231 cells than in less-invasive T47D and MCF-7 cells. The SR141716 antiproliferative effect was not accompanied by apoptosis or necrosis and was characterized by a G_1/S-phase cell cycle arrest, decreased expression of cyclin D and E, and increased levels of cyclin-dependent kinase inhibitor $p27^{KIP1}$. We have also shown that SR141716 exerted a significant antiproliferative action, in vivo, by reducing the volume of xenograft tumors induced by MDA-MB-231 injection in mice. On the other hand, at the concentration range in which we observed the antiproliferative effect in tumor cells, we did not observe evidence of any genotoxic effect on normal cells. Our data also indicate that the SR141716 antiproliferative effect requires lipid raft/caveolae integrity to occur. Indeed, we found that CB1 receptor (CB1R) is completely displaced from lipid rafts in SR141716-treated MDA-MB-231 cells, and cholesterol depletion by methyl-β-cyclodextrin strongly prevented SR141716-mediated antiproliferative effect. Taken together, our results suggest that SR141716 inhibits human breast cancer cell growth via a CB1R lipid raft/caveolae-mediated mechanism.

3.807 Rab14 is part of the early endosomal clathrin-coated TGN microdomain
Proikas-Cezanne, T., Gaugel, A., Frickey, T. and Nordheim, A.
*FEBS Lett., **580**, 5241-5246 (2006)*

Rab14 localizes to the Golgi/TGN and to early endosomes, but its biological function remains unclear. By structural modeling, we identified Rab14-specific residues and established a close relationship between the Rab2/Rab4/Rab14, Rab11/25 and Rab39 sub-groups within the Rab protein family. By quantitative confocal microscopy and by density centrifugation we show that Rab14 is part of the early endosomal AP-1 microdomain. Overexpression of a dominant-negative Rab14 GTP-binding mutant that solely localizes to the Golgi donor compartment accelerated EGF degradation. We suggest that the AP-1 microdomain represents the interconnecting compartment in which Rab14 vesicles cycle between early endosomes and the Golgi cisternae.

3.808 Use of analogs and inhibitors to study the functional significance of protein palmitoylation
Resh, M.D.
*Methods, **40**, 191-197 (2006)*

Covalent attachment of palmitate to proteins is a post-translational modification that exerts diverse effects on protein localization and function. The three key technical approaches required for an investigator to determine the role of palmitoylation of your favorite palmitoylated protein (YFPP) are methods to: (1) detect YFPP palmitoylation; (2) alter or inhibit palmitoylation of YFPP; (3) determine the functional significance of altered YFPP palmitoylation. Here, I describe experimental methods to address these three issues. Both radioactive (radiolabeling with [^{3}H]palmitate or ^{125}I-IC16 palmitate) and non-radioactive (chemical labeling and mass spectrometry) methods to detect palmitoylated proteins are presented. Next, techniques to inhibit protein palmitoylation are described. These include site specific mutagenesis, and treatment of cells with inhibitors of protein palmitoylation, including 2-bromopalmitate, cerulenin, and tunicamycin. Alternative methods to replace palmitate with other fatty acids are also presented. Finally, general approaches to determining the effect of altered palmitoylation status on YFPP association with membranes and lipid rafts, as well as signal transduction, are described.

3.809 Different Routes of Bone Morphogenic Protein (BMP) Receptor Endocytosis Influence BMP Signaling
Hartung, A. et al
*Mol. Cell. Biol., **26(20)**, 7791-7805 (2006)*

Endocytosis is important for a variety of functions in eukaryotic cells, including the regulation of signaling cascades via transmembrane receptors. The internalization of bone morphogenetic protein (BMP) receptor type I (BRI) and type II (BRII) and its relation to signaling were largely unexplored. Here, we demonstrate that both receptor types undergo constitutive endocytosis via clathrin-coated pits (CCPs) but that only BRII undergoes also caveola-like internalization. Using several complementary approaches, we could show that (i) BMP-2-mediated Smad1/5 phosphorylation occurs at the plasma membrane in nonraft regions, (ii) continuation of Smad signaling resulting in a transcriptional response requires endocytosis via the clathrin-mediated route, and (iii) BMP signaling leading to alkaline phosphatase induction initiates from receptors that fractionate into cholesterol-enriched, detergent-resistant membranes. Furthermore, we show that BRII interacts with Eps15R, a constitutive component of CCPs, and with caveolin-1, the marker protein of caveolae. Taken together, the localization of BMP receptors in distinct membrane domains is prerequisite to their taking different endocytosis routes with specific impacts on Smad-dependent and Smad-independent signaling cascades.

3.810 Ultrastructural Analysis of ESCRT Proteins Suggests a Role for Endosome-Associated Tubular–Vesicular Membranes in ESCRT Function
Welsch, S. et al
*Traffic, **7**, 1551-1566 (2006)*

The endosomal sorting complex required for transport (ESCRT) is thought to support the formation of intralumenal vesicles of multivesicular bodies (MVBs). The ESCRT is also required for the budding of HIV and has been proposed to be recruited to the HIV-budding site, the plasma membrane of T cells and MVBs in macrophages. Despite increasing data on the function of ESCRT, the ultrastructural localization of its components has not been determined. We therefore localized four proteins of the ESCRT machinery in human T cells and macrophages by quantitative electron microscopy. All the proteins were found throughout the endocytic pathway, including the plasma membrane, with only around 10 and 3% of the total labeling in the cytoplasm and on the MVBs, respectively. The majority of the labeling (45%) was unexpectedly found on tubular–vesicular endosomal membranes rather than on endosomes themselves. The ESCRT labeling was twice as concentrated on early and late endosomes/lysosomes in macrophages compared with that in T cells, where it was twice more abundant at the plasma membrane. The ESCRT proteins were not redistributed on HIV infection, suggesting that the amount of ESCRT proteins located at the budding site suffices for HIV release. These results represent the first systematic ultrastructural localization of ESCRT and provide insights into its role in uninfected and HIV-infected cells.

3.811 Molecular probes for sensing the cholesterol composition of subcellular organelle membranes
Wang, R. et al
*Biochim. Biophys. Acta, **1761**, 1169-1181 82006)*

Neuroendocrine cells contain two types of secretagogue-regulated acidic compartments: secretory granules (SGs) and synaptic-like microvesicles (SLMVs), which can be identified by acidotropic probes such as acridine orange (AO) and DAMP. We investigated the accumulation of these probes in SGs and SLMVs as a function of glucose levels in the culture media using a pancreatic β-cell line MIN6. AO was accumulated in the low-glucose condition, but not in the high-glucose condition. The AO accumulation correlated well with the SLMV dynamics by glucose and DAMP was localized in the SGs. Because SG membranes are reportedly high in cholesterol, we prepared liposomes with increasing cholesterol levels. AO is well incorporated into liposomes having a 20 to 40 mol% cholesterol composition, whereas DAMP was so in those having over 40 mol% cholesterol levels. Indeed, when cholesterol was depleted from MIN6 SG membranes, DAMP incorporation decreased, instead AO was incorporated. In PC12 cells, AO incorporation into SGs was significant but DAMP incorporation was limited. Consistently, the cholesterol composition was found 37 to 39 mol% in the SG membrane of PC12 cells. We suggest that cholesterol-sensing probes, AO and DAMP, are useful tools for investigating cholesterol compositions in acidic organelle membranes.

3.812 Evidence against Calcium as a Mediator of Mitochondrial Dysfunction during Apoptosis Induced by Arachidonic Acid and Other Free Fatty Acids
Maia, R.C., Culver, C.A. and Laster, S.M.
*J. Immunol., **177**, 6398-6404 (2006)*

Apoptosis is often accompanied by activation of phospholipase A_2, causing release of free fatty acids (FFAs), which in turn are thought to contribute to the loss of mitochondrial transmembrane potential ($\Delta\psi_m$). In these experiments, we asked whether calcium plays a role as an intermediate in this process. A total of 14 FFAs were compared for their ability to cause loss of $\Delta\psi_m$ and for their ability to affect levels of intracellular calcium. Among the FFAs, unsaturated FFAs tended to induce apoptosis while saturated FFAs did not. Arachidonic acid (AA) was most damaging, causing loss of $\Delta\psi_m$ and cell death in 8–10 h while linoleic acid, γ-linolenic acid, and docosapentaenoic also strongly induced apoptosis. Effects of the FFAs on levels of intracellular calcium were very different. Many caused strong calcium responses; however, the ability to induce a strong calcium response was not predictive of ability to induce apoptosis, and overall, we did not find a correlation between apoptosis and calcium induction. Also, verapamil and TMB-8 were able to block the calcium response, but these inhibitors did not prevent loss of $\Delta\psi_m$, indicating that the calcium response is not necessary for FFA-induced loss of $\Delta\psi_m$. In contrast, we found that cyclosporine A could inhibit the AA-induced loss of $\Delta\psi_m$ with both whole cells and isolated mitochondria, confirming that the antimitochondrial effects of FFA can stem from direct effects on the mitochondrial permeability transition pore. Finally, we show that the strong apoptosis-inducing activity of AA may stem from its ability to selectively induce its own release.

3.813 ErbB-4 and TNF-α converting enzyme localization to membrane microd
Thiel, K.W. and Carpenter, G.
*Biochem. Biophys. Res. Comm., **350(3)**, 629-633 (2006)*

Sequential proteolytic processing of ErbB-4 occurs in response to ligand addition. Here, we assess the localization of cleavable and non-cleavable ErbB-4 isoforms to membrane microdomains using three methodologies: (1) Triton X-100-insolubility, (2) Brij98-insolubility, and (3) detergent-free density gradient centrifugation. Whereas ErbB-4 translocated to a Triton X-100-insoluble fraction upon treatment of T47D cells with heregulin, it constitutively associated with a Brij98-insoluble fraction and a lipid raft fraction isolated using detergent-free methodology. Comparison of cleavable and non-cleavable isoforms of ErbB-4 revealed that both ErbB-4 isoforms are constitutively localized to either a Triton X-100-soluble or Brij98-insoluble fraction. In contrast, addition of heregulin resulted in translocation of the cleavable isoform to a detergent-free lipid raft. Tumor necrosis factor-α converting enzyme (TACE), the ectodomain secretase for ErbB-4, was present predominantly in its mature active form in most microdomains analyzed. These data suggest the assembly of ErbB-4 ectodomain cleavage apparatus in a membrane microdomain.

3.814 Targeting β2-microglobulin for induction of tumor apoptosis in human hematological malignancies
Yang, J. et al
*Cancer Cell, **10**, 295-307 (2006)*

We discovered that monoclonal antibodies (mAbs) specific to human β_2-microglobulin (β_2M) induce apoptosis in vitro and were therapeutic in mouse models of myeloma and other hematological tumor cells. Cell death occurred rapidly, without the need for exogenous immunological effector mechanisms. The mAbs induced cell death via recruiting MHC class I molecules to lipid rafts and activating Lyn and PLCγ2, leading to activated JNK and inhibited PI3K/Akt and ERK, compromised mitochondrial integrity, and caspase-9-dependent cascade activation. Although the expression of β_2M on normal hematopoietic cells is a potential safety concern, the mAbs were selective to tumor-transformed cells and did not induce apoptosis of normal cells. Therefore, such mAbs offer the potential for a therapeutic approach to hematological malignancies.

3.815 Protein Misfolding Cyclic Amplification for Diagnosis and Prion Propagation Studies
Castilla, J. et al
*Methods in Enzymol., **412**, 3-21 (2006)*

Diverse human disorders are thought to arise from the misfolding and aggregation of an underlying protein. Among them, prion diseases are some of the most intriguing disorders that can be transmitted by an unprecedented infectious agent, termed prion, composed mainly (if not exclusively) of the misfolded prion protein. The hallmark event in the disease is the conversion of the native prion protein into the disease-

associated misfolded protein. We have recently described a novel technology to mimic the prion conversion process *in vitro*. This procedure, named protein misfolding cyclic amplification (PMCA), conceptually analogous to DNA amplification by polymerase chain reaction (PCR), has important applications for research and diagnosis. In this chapter we describe the rational behind PMCA and some of the many potential applications of this novel technology. We also describe in detail the technical and methodological aspects of PMCA, as well as its application in automatic and serial modes that have been developed with a view to improving disease diagnosis.

3.816 Modulation of GalT1 and SialT1 Sub-Golgi Localization by SialT2 Expression Reveals an Organellar Level of Glycolipid Synthesis Control
Uliana, A.S., Crespo, P.M., Martina, J.A., Daniotti, J.L. and Maccioni, H.J.F.
J. Biol. Chem., ***281(43)***, *32852-32860 (2006)*

Ganglioside glycosyltransferases organize as multienzyme complexes that localize in different sub-Golgi compartments. Here we studied whether in CHO-K1 cells lacking CMP-NeuAc: GM3 sialyltransferase (SialT2), the sub-Golgi localization of UDP-Gal:glucosylceramide beta-1,4-galactosyltransferase (GalT1) and CMP-NeuAc:lactosylceramide sialyltransferase (SialT1) complex is affected when SialT2, another member of this complex, is coexpressed. GalT1 and SialT1 sub-Golgi localization was determined by studying the effect of brefeldin A (BFA) and monensin on the synthesis of glycolipids and on the sub-Golgi localization of GalT1(1-52)-CFP (cyan fluorescent protein) and SialT1(1-54)-YFP (yellow fluorescent protein) chimeras by single cell fluorescence microscopy and by isopycnic subfractionation. We found that BFA, and also monensin, impair the synthesis of glycolipids beyond GM3 ganglioside in wild type (WT) cells but beyond GlcCer in SialT2(+) cells. Although BFA redistributed GalT1-CFP and SialT1-YFP to the endoplasmic reticulum in WT cells, a fraction of these chimeras remained associated with a distal Golgi compartment, enriched in trans Golgi network, and recycling endosome markers in SialT2(+) cells. In BFA-treated cells, the percentage of GalT1-CFP and SialT1-YFP associated with Golgi-like membrane fractions separated by isopycnic subfractionation was higher in SialT2(+) cells than in WT cells. These effects were reverted by knocking down the expression of SialT2 with specific siRNA. Results indicate that sub-Golgi localization of glycosyltransferase complexes may change according to the relative levels of the expression of participating enzymes and reveal a capacity of the organelle to adapt the topology of the glycolipid synthesis machinery to functional states of the cell.

3.817 Kinesin-2 mediates physical and functional interactions between polycystin-2 and fibrocystin
Wu, Y. et al
Hum. Mol. Genet., ***15(22)***, *3280-3292 (2006)*

Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in *PKD1*, encoding polycystin-1 (PC1), or *PKD2* (polycystin-2, PC2). Autosomal recessive PKD (ARPKD) is caused by mutations in *PKHD1*, encoding fibrocystin/polyductin (FPC). No molecular link between ADPKD and ARPKD has been determined. Here, we demonstrated, by yeast two-hybrid and biochemical assays, that KIF3B, a motor subunit of kinesin-2, associates with PC2 and FPC. Co-immunoprecipitation experiments using Madin-Darby canine kidney (MDCK) and inner medullary collecting duct (IMCD) cells and human kidney revealed that PC2 and KIF3B, FPC and KIF3B and, furthermore, PC2 and FPC are endogenously in the same complex(es), though no direct association between the PC2 and FPC intracellular termini was detected. *In vitro* binding and Far Western blot experiments demonstrated that PC2 and FPC are in the same complex only if KIF3B is present, presumably by forming a PC2–KIF3B–FPC complex. This was supported by our observation that altering KIF3B level in IMCD cells by over-expression or siRNA significantly affected complexing between PC2 and FPC. Immunofluorescence experiments showed that PC2, FPC and KIF3B partially co-localized in primary cilia of over-confluent and perinuclear regions of sub-confluent cells. Furthermore, KIF3B mediated functional modulation of purified PC2 channels by FPC in a planer lipid bilayer electrophysiology system. The FPC C-terminus substantially stimulated PC2 channel activity in the presence of KIF3B, whereas FPC or KIF3B alone had no effect. Taken together, we discovered that kinesin-2 is a linker between PC2 and FPC and mediates the regulation of PC2 channel function by FPC. Our study may be important for elucidating common molecular pathways for PKD of different genotypes.

3.818 Lipid Phosphate Phosphatases 1 and 3 Are Localized in Distinct Lipid Rafts
Kai, M. et al
J. Biochem., ***140***, *677-686 (2006)*

Lipid phosphate phosphatases (LPPs), integral membrane proteins with six transmembrane domains, dephosphorylate a variety of extracellular lipid phosphates. Although LPP3 is already known to bind to Triton X-100–insoluble rafts, we here report that LPP1 is also associated with lipid rafts distinct from those harboring LPP3. We found that LPP1 was Triton X-100–soluble, but CHAPS-insoluble in LNCaP cells endogenously expressing LPP1 and several LPP1 cDNA–transfected cells including NIH3T3 fibroblasts. In addition to the non–ionic detergent insolubility, LPP1 further possessed several properties formulated for raft-localizing proteins as follows: first, the CHAPS-insolubility was resistant to the actin-disrupting drug cytochalasin D; second, the CHAPS-insoluble LPP1 floated in an **Optiprep** density gradient; third, the CHAPS insolubility of LPP1 was lost by cholesterol depletion; and finally, the subcellular distribution pattern of LPP1 exclusively overlapped with that of a raft marker, cholera toxin B subunit. Interestingly, confocal microscopic analysis showed that LPP1 was distributed to membrane compartments distinct from those of LPP3. Analysis using various LPP1/LPP3 chimeras revealed that their first extracellular regions determine the different Triton X-100 solubilities. These results indicate that LPP1 and LPP3 are distributed in distinct lipid rafts that may provide unique microenvironments defining their non-redundant physiological functions.

3.819 **Aquaporin-5 water channel in lipid rafts of rat parotid glands**
Ishikawa, Y., Cho, G., Yuan, Z., Inoue, N. and Nakae, Y.
*Biochim. Biophys. Acta, **1758(8)**, 1053-1060 (2006)*

Aquaporin-5 (AQP5), an apical plasma membrane (APM) water channel in salivary glands, lacrimal glands, and airway epithelium, has an important role in fluid secretion. The activation of M3 muscarinic acetylcholine receptors (mAChRs) or α1-adrenoceptors on the salivary glands induces salivary fluid secretion. AQP5 localizes in lipid rafts and activation of the M3 mAChRs or α1-adrenoceptors induced its translocation together with the lipid rafts to the APM in the interlobular ducts of rat parotid glands. This review focuses on the mechanisms of AQP5 translocation together with lipid rafts to the APM in the interlobular duct cells of parotid glands of normal rats and the impairment of AQP5 translocation in diabetes and senescence.

3.820 **Myelin basic protein-dependent plasma membrane reorganization in the formation of myelin**
Fitzner, D. et al
*EMBO J., **25**, 5037-5048 (2006)*

During vertebrate development, oligodendrocytes wrap their plasma membrane around axons to produce myelin, a specialized membrane highly enriched in galactosylceramide (GalC) and cholesterol. Here, we studied the formation of myelin membrane sheets in a neuron–glia co-culture system. We applied different microscopy techniques to visualize lipid packing and dynamics in the oligodendroglial plasma membrane. We used the fluorescent dye Laurdan to examine the lipid order with two-photon microscopy and observed that neurons induce a dramatic lipid condensation of the oligodendroglial membrane. On a nanoscale resolution, using stimulated emission depletion and fluorescence resonance energy transfer microscopy, we demonstrated a neuronal-dependent clustering of GalC in oligodendrocytes. Most importantly these changes in lipid organization of the oligodendroglial plasma membrane were not observed in shiverer mice that do not express the myelin basic protein. Our data demonstrate that neurons induce the condensation of the myelin-forming bilayer in oligodendrocytes and that MBP is involved in this process of plasma membrane rearrangement. We propose that this mechanism is essential for myelin to perform its insulating function during nerve conduction.

3.821 **Very Long-chain Fatty Acid-containing Lipids rather than Sphingolipids per se Are Required for Raft Association and Stable Surface Transport of Newly Synthesized Plasma Membrane ATPase in Yeast**
Gaigg, B., Toulmay, A. and Scheiter, R.
*J. Biol. Chem., **281(45)**, 34135-34145 (2006)*

The proton-pumping H^+-ATPase, Pma1p, is an abundant and very long lived polytopic protein of the yeast plasma membrane. Pma1p constitutes a major cargo of the secretory pathway and thus serves as a model to study plasma membrane biogenesis. Pma1p associates with detergent-resistant membrane domains (lipid "rafts") already in the ER, and a lack of raft association correlates with mistargeting of the protein to the vacuole, where it is degraded. We are analyzing the role of specific lipids in membrane domain formation and have previously shown that surface transport of Pma1p is independent of newly synthesized sterols but that sphingolipids with C26 very long chain fatty acid are crucial for raft association and surface transport

of Pma1p (Gaigg, B., Timischl, B., Corbino, L., and Schneiter, R. (2005) *J. Biol. Chem.* 280, 22515-22522). We now describe a more detailed analysis of the function that sphingolipids play in this process. Using a yeast strain in which the essential function of sphingolipids is substituted by glycerophospholipids containing C26 very long chain fatty acids, we find that sphingolipids *per se* are dispensable for raft association and surface delivery of Pma1p but that the C26 fatty acid is crucial. We thus conclude that the essential function of sphingolipids for membrane domain formation and stable surface delivery of Pma1p is provided by the C26 fatty acid that forms part of the yeast ceramide.

3.822 Transactivation of Sphingosine 1-Phosphate Receptors Is Essential for Vascular Barrier Regulation: NOVEL ROLE FOR HYALURONAN AND CD44 RECEPTOR FAMILY
Singleton, P.A., Dudek, S.M., Ma, S-F. and Garcia, J.G.N.
J. Biol. Chem., ***281(45)***, *34381-34393 (2006)*

The role for hyaluronan (HA) and CD44 in vascular barrier regulation is unknown. We examined high and low molecular weight HA (HMW-HA, ~1,000 kDa; LMW-HA, ~2.5 kDa) effects on human transendothelial monolayer electrical resistance (TER). HMW-HA increased TER, whereas LMW-HA induced biphasic TER changes ultimately resulting in EC barrier disruption. HMW-HA induced the association of the CD44s isoform with, and AKT-mediated phosphorylation of, the barrier-promoting sphingosine 1-phosphate receptor ($S1P_1$) within caveolin-enriched lipid raft microdomains, whereas LMW-HA induced brief CD44s association with $S1P_1$ followed by sustained association of the CD44v10 isoform with, and Src and ROCK 1/2-mediated phosphorylation of, the barrier-disrupting $S1P_3$ receptor. HA-induced EC cytoskeletal reorganization and TER alterations were abolished by either disruption of lipid raft formation, CD44 blocking antibody or siRNA-mediated reductions in expression of CD44 isoforms. Silencing $S1P_1$, AKT1, or Rac1 blocked the barrier enhancing effects of HA whereas silencing $S1P_3$, Src, ROCK1/2, or RhoA blocked the barrier disruption induced by LMW-HA. In summary, HA regulates EC barrier function through novel differential CD44 isoform interaction with S1P receptors, S1P receptor transactivation, and RhoA/Rac1 signaling to the EC cytoskeleton.

3.823 Acylation of CD44 and Its Association with Lipid Rafts Are Required for Receptor and Hyaluronan Endocytosis
Thankamony, S.P. and Knudson, W.
J. Biol. Chem., ***281(45)***, *34601-34609 (2006)*

CD44 is a cell surface receptor for the extracellular matrix macromolecule hyaluronan. In addition, CD44 mediates the endocytosis of hyaluronan leading to its subsequent degradation within lysosomes. Using model systems of COS-7 and Flp-293 cells, we demonstrate that the association of CD44 with lipid rafts is essential for the endocytosis of hyaluronan but not the extracellular binding. Further, we demonstrate that palmitoylation of CD44 on two highly conserved cysteine residues is essential for the association with lipid rafts as determined by density gradient ultracentrifugation. Mutations of either cysteine residues or pretreatment of cells with the palmitic acid analog 2-bromopalmitate, reduced the [^{3}H]palmitic acid incorporation into CD44 and prevented CD44-lipid rafts association. Preventing CD44 palmitoylation had no effect on the binding of hyaluronan but inhibited hyaluronan internalization. The turnover of the CD44 receptor itself was also affected by blocking its association with lipid rafts. Using cycloheximide to prevent *de novo* protein synthesis, palmitoylation-deficient cysteine mutants underwent slower turnover from cell surface compared with the palmitoylation-intact wild type, as determined by immunofluorescence and Western blotting. These results indicate that palmitoylation of CD44 is a critical driving determinant to CD44 association with lipid rafts and, concomitantly, the rates of hyaluronan endocytosis and CD44 turnover from cell surface.

3.824 Perturbed Interactions of Mutant Proteolipid Protein/DM20 with Cholesterol and Lipid Rafts in Oligodendroglia: Implications for Dysmyelination in Spastic Paraplegia
Krämer-Alber, E-M., Gehrig-Burger, K., Thiele, C., Trotter, J. and Nave, K-A.
J. Neuroscience, ***26(45)***, *11743-11752 (2006)*

Missense mutations in the human *PLP1* gene lead to dysmyelinating diseases with a broad range of clinical severity, ranging from severe Pelizaeus–Merzbacher disease (PMD) to milder spastic paraplegia type 2 (SPG-2). The molecular pathology has been generally attributed to endoplasmic reticulum (ER) retention of misfolded proteolipid protein (PLP) (and its splice isoform DM20) and induction of the unfolded protein response. As opposed to previous studies of heterologous expression systems, we have analyzed PLP/DM20 trafficking in oligodendroglial cells, thereby revealing differences between PMD and SPG-2-

associated PLP/DM20 isoforms. PLP^{A242V} and $DM20^{A242V}$ (*jimpy-msd* in mice), associated with severe PMD-like phenotype *in vivo*, were not only retained in the ER but also interfered with oligodendroglial process formation. In contrast, glial cells expressing SPG-2-associated PLP^{I186T} or $DM20^{I186T}$ (*rumpshaker* in mice) developed processes, and mutant PLP/DM20 reached a late endosomal/lysosomal compartment. Unexpectedly, PLP/DM20 with either substitution exhibited impaired cholesterol binding, and the association with lipid raft microdomains was strongly reduced. Turnover analysis demonstrated that mutant PLP was rapidly degraded in oligodendroglial cells, with half-lives for PLP > PLP^{I186T} > PLP^{A242V}. Protein degradation was specifically sensitive to proteasome inhibition, although PLP/$DM20^{I186T}$ degradation was also affected by inhibition of lysosomal enzymes. We conclude that, in addition to ER retention and unfolded protein response (UPR) induction, impaired cholesterol binding and lipid raft association are characteristic cellular defects of *PLP1*-missense mutations. Mutant protein is rapidly cleared and does not accumulate in oligodendroglial cells. Whereas UPR-induced cell death governs the PMD phenotype of the *msd* mutation, we propose that impaired cholesterol and lipid raft interaction of the *rsh* protein may contribute to the dysmyelination observed in SPG-2.

3.825 Quantitative Proteomics Analysis of Detergent-resistant Membranes from Chemical Synapses: Evidence for Cholesterol as Spatial Organizer of Synaptic Vesicle Cycling
Jia, J-y. et al
*Mol. Cell. Proteomics, **5**, 2060-2071 (2006)*

Synaptic vesicles (SVs) in the central nervous system upon stimulation undergo rapid calcium-triggered exoendocytic cycling within the nerve terminal that at least in part depends on components of the clathrin- and dynamin-dependent endocytosis machinery. How exocytic SV fusion and endocytic retrieval are temporally and spatially coordinated is still an open question. One possibility is that specialized membrane microdomains characterized by their high content in membrane cholesterol may assist in the spatial coordination of synaptic membrane protein recycling. Quantitative proteomics analysis of detergent-resistant membranes (DRMs) isolated from rat brain synapses or cholesterol-depleted control samples by liquid chromatography-tandem mass spectrometry identified a total of 159 proteins. Among these 122 proteins were classified as cholesterol-dependent DRM or DRM-associated proteins, many of which with proven or hypothesized functions in exoendocytic vesicle cycling including clathrin, the clathrin adaptor complex AP-2, and a variety of SV proteins. In agreement with this, SV membrane and endocytic proteins displayed a partial resistance to extraction with cold Triton X-100 in cultured rat hippocampal neurons where they co-localized with labeled cholera toxin B, a marker for cholesterol-enriched DRMs. Moreover SV proteins formed cholesterol-dependent complexes in CHAPS-extracted synaptic membrane lysates. Our combined data suggest that lipid microdomains may act as spatial coordinators for exoendocytic vesicle cycling at synapses.

3.826 Cellular uptake of fatty acids driven by the ER-localized acyl-CoA synthetase FATP4
Milger, K. et al
*J. Cell Sci., **119**, 4678-4688 (2006)*

Long-chain fatty acids are important metabolites for the generation of energy and the biosynthesis of lipids. The molecular mechanism of their cellular uptake has remained controversial. The fatty acid transport protein (FATP) family has been named according to its proposed function in mediating this process at the plasma membrane. Here, we show that FATP4 is in fact localized to the endoplasmic reticulum and not the plasma membrane as reported previously. Quantitative analysis confirms the positive correlation between expression of FATP4 and uptake of fatty acids. However, this is dependent on the enzymatic activity of FATP4, catalyzing the esterification of fatty acids with CoA. Monitoring fatty acid uptake at the single-cell level demonstrates that the ER localization of FATP4 is sufficient to drive transport of fatty acids. Expression of a mitochondrial acyl-CoA synthetase also enhances fatty acid uptake, suggesting a general relevance for this mechanism. Our results imply that cellular uptake of fatty acids can be regulated by intracellular acyl-CoA synthetases. We propose that the enzyme FATP4 drives fatty acid uptake indirectly by esterification. It is not a transporter protein involved in fatty acid translocation at the plasma membrane.

3.827 Identification of a Novel Apical Sorting Motif and Mechanism of Targeting of the M2 Muscarinic Acetylcholine Receptor
Chmelar, R.S. and Nathanson, N.M.
*J. Biol. Chem., **281(46)**, 35381-35396 (2006)*

Previous studies have shown that the M_2 receptor is localized at steady state to the apical domain in Madin-Darby canine kidney (MDCK) epithelial cells. In this study, we identify the molecular determinants governing the localization and the route of apical delivery of the M_2 receptor. First, by confocal analysis of a transiently transfected glycosylation mutant in which the three putative glycosylation sites were mutated, we determined that *N*-glycans are not necessary for the apical targeting of the M_2 receptor. Next, using a chimeric receptor strategy, we found that two independent sequences within the M_2 third intracellular loop can confer apical targeting to the basolaterally targeted M_4 receptor, Val^{270}-Lys^{280} and Lys^{280}-Ser^{350}. Experiments using Triton X-100 extraction followed by **OptiPrep**™ density gradient centrifugation and cholera toxin β-subunit-induced patching demonstrate that apical targeting is not because of association with lipid rafts. ^{35}S-Metabolic labeling experiments with domain-specific surface biotinylation as well as immunocytochemical analysis of the time course of surface appearance of newly transfected confluent MDCK cells expressing FLAG-M_2-GFP demonstrate that the M_2 receptor achieves its apical localization after first appearing on the basolateral domain. Domain-specific application of tannic acid of newly transfected cells indicates that initial basolateral plasma membrane expression is required for subsequent apical localization. This is the first demonstration that a G-protein-coupled receptor achieves its apical localization in MDCK cells via transcytosis.

3.828 The cell biology of HIV-1 and other retroviruses
Freed, E.O. and Mouland, A.J.
*Retrovirology, **3(77)**, 1-10 (2006)*

In recognition of the growing influence of cell biology in retrovirus research, we recently organized a Summer conference sponsored by the American Society for Cell Biology (ASCB) on the Cell Biology of HIV-1 and other Retroviruses (July 20–23, 2006, Emory University, Atlanta, Georgia). The meeting brought together a number of leading investigators interested in the interplay between cell biology and retrovirology with an emphasis on presentation of new and unpublished data. The conference was arranged from early to late events in the virus replication cycle, with sessions on viral fusion, entry, and transmission; post-entry restrictions to retroviral infection; nuclear import and integration; gene expression/regulation of retroviral Gag and genomic RNA; and assembly/release. In this review, we will attempt to touch briefly on some of the highlights of the conference, and will emphasize themes and trends that emerged at the meeting.

3.829 Study of proteins associated with the Eimeria tenella refractile body by a proteomic approach
De Venevelles, P. et al
*Int. J. Parasitol., **36(13)**, 1399-1407 (2006)*

Refractile bodies (RB), whose function is still unknown, are specific structures of Eimeriidae parasites. In order to study their proteome, RB were purified from *Eimeria tenella* sporozoites by a new procedure using a reversible fixation followed by centrifugation. RB proteins were resolved by two-dimensional electrophoresis. Around 76 and 89 spots were detected on RB two-dimensional gels using gradients in the 3–10 and 4–7 range, respectively. RB proteins were located mainly between pH 5 and 7. RB gels were then compared with previously established maps of the entire sporozoite proteome. Proteins appearing in new spots were identified by mass spectrometry. Thirty protein isoforms were located in RB. Added to the already known RB proteins such as Eimepsin and SO7′, the new RB proteins were defined as haloacid dehalogenase, hydrolase, subtilase, lactacte dehydrogenase or ubiquitin family proteins. The RB proteome analysis confirmed the hypothesis that this structure is a reservoir for proteins necessary to invasion but also suggests that RB have energetic and metabolic functions.

3.830 Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response
Baumann, S.J. and Kuehn, M.J.
*Microbes and Infection, **8(9-10)**, 2400-2408 (2006)*

Considerable lung injury results from the inflammatory response to *Pseudomonas aeruginosa* infections in patients with cystic fibrosis (CF). The *P. aeruginosa* laboratory strain PAO1, an environmental isolate, and isolates from CF patients were cultured in vitro and outer membrane vesicles from those cultures were quantitated, purified, and characterized. Vesicles were produced throughout the growth phases of the culture and vesicle yield was strain-independent. Strain-dependent differences in the protein composition of vesicles were quantitated and identified. The aminopeptidase PaAP (PA2939) was highly enriched in vesicles from CF isolates. Vesicles from all strains elicited IL-8 secretion by lung epithelial cells. These

results suggest that *P. aeruginosa* colonizing the CF lung may produce vesicles with a particular composition and that the vesicles could contribute to inflammation.

3.831 Specific and distinct determinants mediate membrane binding and lipid raft incorporation of HIV-1SF2 Nef
Giese, S.I. et al
Virology, ***355(2)****, 175-191 (2006)*

Membrane association is believed to be a prerequisite for the biological activity of the HIV-1 pathogenicity factor Nef. Attachment to cellular membranes as well as incorporation into detergent-insoluble microdomains (lipid rafts) require the N-terminal myristoylation of Nef. However, this modification is not sufficient for sustained membrane association and a specific raft-targeting signal for Nef has not yet been identified. Using live cell confocal microscopy and membrane fractionation analyses, we found that the N-terminal anchor domain (aa 1–61) is necessary and sufficient for efficient membrane binding of Nef from HIV-1_{SF2}. Within this domain, highly conserved lysine and arginine residues significantly contributed to Nef's membrane association and localization. Plasma membrane localization of Nef was also governed by an additional membrane-targeting motif between residues 40 and 61. Importantly, two lysines at positions 4 and 7 were not essential for the overall membrane association but critically contributed to Nef's incorporation into lipid raft domains. Cell surface receptor downmodulation was largely unaffected by mutations of all N-terminal basic residues, while the association of Nef with Pak2 kinase activity and its ability to augment virion infectivity correlated with its lysine-mediated raft incorporation. In contrast, all basic residues were required for efficient HIV-1 replication in primary human T lymphocytes but did not contribute to the incorporation of Nef into HIV-1 virions. Together, these results unravel that Nef's membrane association is governed by a complex pattern of signature motifs that differentially contribute to individual Nef activities. The identification of a critical raft targeting determinant and the functional characterization of a membrane-bound, non-raft-associated Nef variant indicate raft incorporation as a regulatory mechanism that determines the biological activity of distinct subpopulations of Nef in HIV-infected cells.

3.832 Dual regulation of translation initiation and peptide chain elongation during BDNF-induced LTP in vivo: evidence for compartment-specific translation control
Kanhema, T. et al
J. Neurochem., ***99****, 1328-1337 (2006)*

Protein synthesis underlying activity-dependent synaptic plasticity is controlled at the level of mRNA translation. We examined the dynamics and spatial regulation of two key translation factors, eukaryotic initiation factor 4E (eIF4E) and elongation factor-2 (eEF2), during long-term potentiation (LTP) induced by local infusion of brain-derived neurotrophic factor (BDNF) into the dentate gyrus of anesthetized rats. BDNF-induced LTP led to rapid, transient phosphorylation of eIF4E and eEF2, and enhanced expression of eIF4E protein in dentate gyrus homogenates. Infusion of the extracellular signal-regulated kinase (ERK) inhibitor U0126 blocked BDNF-LTP and modulation of the translation factor activity and expression. Quantitative immunohistochemical analysis revealed enhanced staining of phospho-eIF4E and total eIF4E in dentate granule cells. The *in vitro* synaptodendrosome preparation was used to isolate the synaptic effects of BDNF in the dentate gyrus. BDNF treatment of synaptodendrosomes elicited rapid, transient phosphorylation of eIF4E paralleled by enhanced expression of α-calcium/calmodulin-dependent protein kinase II. In contrast, BDNF had no effect on eEF2 phosphorylation state in synaptodendrosomes. The results demonstrate rapid ERK-dependent regulation of the initiation and elongation steps of protein synthesis during BDNF-LTP *in vivo*. Furthermore, the results suggest a compartment-specific regulation in which initiation is selectively enhanced by BDNF at synapses, while both initiation and elongation are modulated at non-synaptic sites.

3.833 The heterogeneity of NaPi protein dynamics and NaPi cotransport activity in renal brush border membranes
Bliane, J.T. et al
FASEB J., ***20****, A59 (2006)*

Alterations in renal proximal tubule brush border membrane (BBM) cholesterol, sphingomyelin, and glycosphingolipid content play an important role in regulating the activity of the sodium-phosphate cotransporter (NaPi). The molecular mechanisms of how alterations in lipid composition may modulate NaPi activity are not known. We have fractionated BBM prepared from rat kidney using detergent-free

density gradient ultracentrifugation (**OptiPrep**). We have found that the NaPi protein preferentially partitions into lipid rafts. To determine the potential consequences of the partitioning of the NaPi transporter in these lipid domains we have i) measured NaPi transport activity and ii) used fluctuation correlation spectroscopy (FCS) methods to determine NaPi diffusion. Partitioning of NaPi protein into lipid rafts results in i) decreased NaPi cotransport activity and ii) decreased diffusion of NaPi protein. Similar results were obtained in BBM isolated from the superficial cortex (SC) versus the juxtamedullary cortex (JMC). The three-fold decrease in NaPi cotransport activity in JMC-BBM is associated with a 3-fold decrease in NaPi protein diffusion. Our results therefore indicate that partitioning of NaPi protein into lipid rafts results in impairment of its activity and diffusion.

3.834 **APPL1 Associates with TrkA and GIPC1 and Is Required for Nerve Growth Factor-Mediated Signal Transduction**
Lin, D.C. et al
Mol. Cell. Biol., ***26(23)***, *8928-8941 (2006)*

The neurotrophin receptor TrkA plays critical roles in the nervous system by recruiting signaling molecules that activate pathways required for the growth and survival of neurons. Here, we report APPL1 as a TrkA-associated protein. APPL1 and TrkA coimmunoprecipitated in sympathetic neurons. We have identified two routes through which this association can occur. APPL1 was isolated as a binding partner for the TrkA-interacting protein GIPC1 from rat brain lysate by mass spectrometry. The PDZ domain of GIPC1 directly engaged the C-terminal sequence of APPL1. This interaction provides a means through which APPL1 may be recruited to TrkA. In addition, the APPL1 PTB domain bound to TrkA, indicating that APPL1 may associate with TrkA independently of GIPC1. Isolation of endosomal fractions by high-resolution centrifugation determined that APPL1, GIPC1, and phosphorylated TrkA are enriched in the same fractions. Reduction of APPL1 or GIPC1 protein levels suppressed nerve growth factor (NGF)-dependent MEK, extracellular signal-regulated kinase, and Akt activation and neurite outgrowth in PC12 cells. Together, these results indicate that GIPC1 and APPL1 play a role in TrkA function and suggest that a population of endosomes bearing a complex of APPL1, GIPC1, and activated TrkA may transmit NGF signals.

3.835 **Molecular Anatomy of a Trafficking Organelle**
Takamori, S. et al
Cell, ***127***, *831-846 (2006)*

Membrane traffic in eukaryotic cells involves transport of vesicles that bud from a donor compartment and fuse with an acceptor compartment. Common principles of budding and fusion have emerged, and many of the proteins involved in these events are now known. However, a detailed picture of an entire trafficking organelle is not yet available. Using synaptic vesicles as a model, we have now determined the protein and lipid composition; measured vesicle size, density, and mass; calculated the average protein and lipid mass per vesicle; and determined the copy number of more than a dozen major constituents. A model has been constructed that integrates all quantitative data and includes structural models of abundant proteins. Synaptic vesicles are dominated by proteins, possess a surprising diversity of trafficking proteins, and, with the exception of the V-ATPase that is present in only one to two copies, contain numerous copies of proteins essential for membrane traffic and neurotransmitter uptake.

3.836 **ATP-binding Cassette Transporter A1 Expression Disrupts Raft Membrane Microdomains through Its ATPase-related Functions**
Landry, Y.D. et al
J. Biol. Chem., ***281(47)***, *36091-36101 (2006)*

ATP-binding cassette transporter A1 (ABCA1) is known to mediate cholesterol efflux to lipid-poor apolipoprotein A-I. In addition, ABCA1 has been shown to influence functions of the plasma membrane, such as endocytosis and phagocytosis. Here, we report that ABCA1 expression results in a significant redistribution of cholesterol and sphingomyelin from rafts to non-rafts. Caveolin, a raft/caveolae marker also redistributes from punctate caveolae-like structures to the general area of the plasma membrane upon ABCA1 expression. Furthermore, we observed significant reduction of Akt activation in ABCA1-expressing cells, consistent with raft disruption. Cholesterol content in the plasma membrane is, however, not altered. Moreover, we provide evidence that a non-functional ABCA1 with mutation in an ATP-binding domain, A937V, fails to redistribute cholesterol, sphingomyelin, or caveolin. A937V also fails to influence Akt activation. Finally, we show that apolipoprotein A-I preferentially associates with non-raft

membranes in ABCA1-expressing cells. Our results thus demonstrate that ABCA1 causes a change in overall lipid packing of the plasma membrane, likely through its ATPase-related functions. Such reorganization by ABCA1 effectively expands the non-raft membrane fractions and, consequentially, pre-conditions cells for cholesterol efflux.

3.837 Localization and in vitro binding studies suggest that the cytoplasmic/nuclear tobacco lectin can interact in situ with high-mannose and complex N-glyc
Lannoo, N. et al
FEBS Lett., ***580(27)***, *6329-6337 (2006)*

The possible in vivo interaction of the *Nicotiana tabacum* agglutinin (Nictaba) with endogenous glycoproteins was corroborated using a combination of confocal/electron microscopy of an EGFP-Nictaba fusion protein expressed in tobacco Bright Yellow-2 (BY-2) cells and biochemical analyses. In vitro binding studies demonstrated that the expressed EGFP-Nictaba possesses carbohydrate-binding activity. Microscopic analyses confirmed the previously reported cytoplasmic/nuclear location of Nictaba in jasmonate-treated tobacco leaves and provided evidence for the involvement of a nuclear localization signal-dependent transport mechanism. In addition, it became evident that the lectin is not uniformly distributed over the nucleus and the cytoplasm of BY-2 cells. Far Western blot analysis of extracts from whole BY-2 cells and purified nuclei revealed that Nictaba interacts in a glycan inhibitable way with numerous proteins including many nuclear proteins. Enzymatic deglycosylation with PNGase F indicated that the observed interaction depends on the presence of *N*-glycans. Glycan array screening, which showed that Nictaba exhibits a strong affinity for high-mannose and complex *N*-glycans, provided a reasonable explanation for this observation. The cytoplasmic/nuclear localization of a plant lectin that has a high affinity for high-mannose and complex *N*-glycans and specifically interacts with conspecific glycoproteins suggests that *N*-glycosylated proteins might be more important in the cytoplasm and nucleus than is currently believed.

3.838 The role of myristoylation in the membrane association of the Lassa virus matrix protein Z
Strecker, T. et al
Virology, J., ***3***, *93 (2006)*
The Z protein is the matrix protein of arenaviruses and has been identified as the main driving force for budding. Both LCMV and Lassa virus Z proteins bud from cells in the absence of other viral proteins as enveloped virus-like particles. Z accumulates near the inner surface of the plasma membrane where budding takes place. Furthermore, biochemical data have shown that Z is strongly membrane associated. The primary sequence of Z lacks a typical transmembrane domain and until now it is not understood by which mechanism Z is able to interact with cellular membranes. In this report, we analyzed the role of N-terminal myristoylation for the membrane binding of Lassa virus Z. We show that disruption of the N-terminal myristoylation signal by substituting the N-terminal glycine with alanine (Z-G2A mutant) resulted in a significant reduction of Z protein association with cellular membranes. Furthermore, removal of the myristoylation site resulted in a relocalization of Z from a punctuate distribution to a more diffuse cellular distribution pattern. Finally, treatment of Lassa virus-infected cells with various myristoylation inhibitors drastically reduced efficient Lassa virus replication. Our data indicate that myristoylation of Z is critical for its binding ability to lipid membranes and thus, for effective virus budding.

3.839 A Membrane-proximal Tetracysteine Motif Contributes to Assembly of CD3$\delta\epsilon$ and CD3$\gamma\epsilon$ Dimers with the T Cell Receptor
Xu, C., Call, M.E. and Wucherpfennig, K.W.
J. Biol. Chem., ***281(48)***, *36977-36984 (2006)*

Assembly of the T cell receptor (TCR) with its dimeric signaling modules, CD3$\delta\epsilon$, CD3$\gamma\epsilon$, and $\zeta\zeta$, is organized by transmembrane (TM) interactions. Each of the three assembly steps requires formation of a three-helix interface involving one particular basic TCR TM residue and two acidic TM residues of the respective signaling dimer. The extracellular domains of CD3$\delta\epsilon$ and CD3$\gamma\epsilon$ contribute to assembly, but TCR interaction sites on CD3 dimers have not been defined. The structures of the extracellular domains of CD3$\delta\epsilon$ and CD3$\gamma\epsilon$ demonstrated parallel β-strands ending at the first cysteine in the C*XX*C*X*E*XXX* motif present in the stalk segment of each CD3 chain. Mutation of the membrane-proximal cysteines impaired assembly of either CD3 dimer with TCR, and little complex was isolated when all four membrane-proximal cysteines were mutated to alanine. These mutations had, however, no discernable effect on CD3$\delta\epsilon$ or CD3$\gamma\epsilon$ dimerization. CD3$\delta\epsilon$ assembled with a TCRα mutant that lacked both immunoglobulin domains, but shortening of the TCRα connecting peptide reduced assembly, consistent with membrane-

proximal TCRα-CD3$\delta\epsilon$ interactions. Chelation of divalent cations did not affect assembly, indicating that coordination of a cation by the tetracysteine motif was not required. The membrane-proximal cysteines were within close proximity but only formed covalent CD3 dimers when one cysteine was mutated. The four cysteines may thus form two intrachain disulfide bonds integral to the secondary structure of CD3 stalk regions. The three-chain interaction theme first established for the TM domains thus extends into the membrane-proximal domains of TCRα-CD3$\delta\epsilon$ and TCRβ-CD3$\gamma\epsilon$.

3.840 Human cytosolic sulfotransferase 2B1: Isoform expression, tissue specificity and subcellular localization

Falany, C.N., He, D., Dumas, N., Frost, A.R. and Falany, J.L.
J. Steroid Biochem. Mol. Biol., ***102(1-5)***, *214-221 (2006)*

Sulfation is an important Phase II conjugation reaction involved in the synthesis and metabolism of steroids in humans. Two different isoforms (2B1a and 2B1b) are encoded by the sulfotransferase (SULT) 2B1 gene utilizing different start sites of transcription resulting in the incorporation of different first exons. SULT2B1a and SULT2B1b are 350 and 365 amino acids in length, respectively, and the last 342 aa are identical. Message for both SULT2B1 isoforms is present in human tissues although SULT2B1b message is generally more abundant. However, to date only SULT2B1b protein has been detected in human tissues or cell lines. SULT2B1b is localized in the cytosol and/or nuclei of human cells. A unique 3′-extension of SULT2B1b is required for nuclear localization in human BeWo placental choriocarcinoma cells. Nuclear localization is stimulated by forskolin treatment in BeWo cells and serine phosphorylation has been identified in the 3′-extension. SULT2B1b is selective for the sulfation of 3β-hydroxysteroids such as dehydroepiandrosterone and pregnenolone, and may also have a role in cholesterol sulfation in human skin. The substrate specificity, nuclear localization, and tissue localization of SULT2B1b suggest a role in regulating the responsiveness of cells to adrenal androgens via their direct inactivation or by preventing their conversion to more potent androgens and estrogens.

3.841 Respiratory Syncytial Virus F Envelope Protein Associates with Lipid Rafts without a Requirement for Other Virus Proteins

Fleming, E.H., Kolokoltsov, A.A., Davey, R.A., Nichols, J.E. and Roberts Jr., N.J.
J. Virol., ***80(24)***, *12160-12170 (2006)*

Like many enveloped viruses, human respiratory syncytial virus (RSV) assembles at and buds from lipid rafts. Translocation of the envelope proteins to these membrane subdomains is essential for production of infectious virus, but the targeting mechanism is poorly understood and it is not known if other virus proteins are required. Here we demonstrate that F protein of RSV intrinsically targets to lipid rafts without a requirement for any other virus protein, including the SH and G envelope proteins. Recombinant virus deficient in SH and G but retaining F protein expression was used to demonstrate that F protein still localized in rafts in both A549 and HEp-2 cells. Expression of a recombinant F gene by use of plasmid vectors demonstrated that F contains its own targeting domain and localized to rafts in the absence of other virus proteins. The domain responsible for translocation was then mapped. Unlike most other virus envelope proteins, F is unusual since the target signal is not contained within the cytoplasmic domain nor did it involve fatty acid modified residues. Furthermore, exchange of the transmembrane domain with that of the vesicular stomatitis virus G protein, a nonraft protein, did not alter F protein raft localization. Taken together, these data suggest that domains present in the extracellular portion of the protein are responsible for lipid raft targeting of the RSV F protein.

3.842 Transition of Galactosyltransferase 1 from Trans-Golgi Cisterna to the Trans-Golgi Network Is Signal Mediated

Schaub, B.E., Berger, B., Berger, E.G. and Rohrer, J.
Mol. Biol. Cell, ***17***, *5153-5162 (2006)*

The Golgi apparatus (GA) is the organelle where complex glycan formation takes place. In addition, it is a major sorting site for proteins destined for various subcellular compartments or for secretion. Here we investigate β1,4-galactosyltransferase 1 (galT) and α2,6-sialyltransferase 1 (siaT), two *trans*-Golgi glycosyltransferases, with respect to their different pathways in monensin-treated cells. Upon addition of monensin galT dissociates from siaT and the GA and accumulates in swollen vesicles derived from the *trans*-Golgi network (TGN), as shown by colocalization with TGN46, a specific TGN marker. We analyzed various chimeric constructs of galT and siaT by confocal fluorescence microscopy and time-lapse videomicroscopy as well as **Optiprep** density gradient fractionation. We show that the first 13 amino acids

of the cytoplasmic tail of galT are necessary for its localization to swollen vesicles induced by monensin. We also show that the monensin sensitivity resulting from the cytoplasmic tail can be conferred to siaT, which leads to the rapid accumulation of the galT–siaT chimera in swollen vesicles upon monensin treatment. On the basis of these data, we suggest that cycling between the *trans*-Golgi *cisterna* and the *trans*-Golgi network of galT is signal mediated.

3.843 PER1 Is Required for GPI-Phospholipase A2 Activity and Involved in Lipid Remodeling of GPI-anchored Proteins

Fujita, M., Umemura, M., Yoko-o, T. and Jigami, Y.
*Mol. Biol. Cell, **17**, 5253-5264 (2006)*

Glycosylphoshatidylinositol (GPI) anchors are remodeled during their transport to the cell surface. Newly synthesized proteins are transferred to a GPI anchor, consisting of diacylglycerol with conventional C16 and C18 fatty acids, whereas the lipid moiety in mature GPI-anchored proteins is exchanged to either diacylglycerol containing a C26:0 fatty acid in the *sn*-2 position or ceramide in *Saccharomyces cerevisiae*. Here, we report on *PER1*, a gene encoding a protein that is required for the GPI remodeling pathway. We found that GPI-anchored proteins could not associate with the detergent-resistant membranes in *per1*Δ cells. In addition, the mutant cells had a defect in the lipid remodeling from normal phosphatidylinositol (PI) to a C26 fatty acid–containing PI in the GPI anchor. In vitro analysis showed that *PER1* is required for the production of lyso-GPI, suggesting that Per1p possesses or regulates the GPI-phospholipase A_2 activity. We also found that human *PERLD1* is a functional homologue of *PER1*. Our results demonstrate for the first time that *PER1* encodes an evolutionary conserved component of the GPI anchor remodeling pathway, highlighting the close connection between the lipid remodeling of GPI and raft association of GPI-anchored proteins..

3.844 Enhanced Amyloidogenic Metabolism of the Amyloid β-Protein Precursor in the X11L-deficient Mouse Brain

Sano, Y. et al
*J. Biol. Chem., **281(49)**, 37853-37860 (2006)*

X11L, a neuronal adaptor protein, associates with the cytoplasmic domain of APP and suppresses APP cellular metabolism. APP is the precursor of Aβ, whose metabolism is strongly implicated in Alzheimer disease pathogenesis. To examine the roles of X11L function in APP metabolism, including the generation of Aβ in the brain, we produced X11L-deficient mutant mice on the C57BL/6 background. The mutant mice did not exhibit histopathological alterations or compensatory changes in the expression of other X11 family proteins, X11 and X11L2. The expression level and distribution of APP in the brain of mutant mice were also identical to those in wild-type mice. However, in the hippocampus, where substantial levels of X11L and APP are expressed, the mutant mice exhibited a significant increase in the level of the C-terminal fragments of APP produced by cleavage with β-secretase but not α-secretase. The levels of Aβ were increased in the hippocampus of aged mutant mice as compared with age-matched controls. These observations clearly indicate that X11L suppresses the amyloidogenic but not amyloidolytic processing of APP in regions of the brain such as the hippocampus, which express significant levels of X11L.

3.845 Studies of Optineurin, a Glaucoma Gene: Golgi Fragmentation and Cell Death from Overexpression of Wild-Type and Mutant Optineurin in Two Ocular Cell Types

Park, B-C., Shen, X., Samaraweera, M. and Yue, B.Y.J.T.
*Am. J: Pathol., **169(6)**, 1976-1989 (2006)*

Optineurin (OPTN) has recently been linked to glaucoma, a major cause of blindness worldwide. Mutations in OPTN such as $Glu^{50}\rightarrow$Lys (E50K) have been reported in patients, particularly those with normal pressure glaucoma. Here, we show that the endogenous OPTN was not secreted in two ocular cell types, human trabecular meshwork and retinal pigment epithelial cells. It localized instead in the cytoplasm in a diffuse pattern without a distinct association with the Golgi apparatus. When overexpressed, however, wild-type OPTN-green fluorescent protein (GFP) formed foci especially around the Golgi, colocalizing partially with the common endocytic pathway marker transferrin receptor in both cell types. Fragmentation of the Golgi was also observed. On nocodazole treatment, the OPTN foci were dispersed into the cytoplasm. Overexpression of mutant $OPTN_{E50K}$-GFP resulted in a greater number ($\boldsymbol{P} < 0.0055$) and size of the foci, compared with the wild type, and the Golgi alteration was potentiated. Cell loss observed in OPTN-expressing cultures was also more pronounced in $OPTN_{E50K}$-GFP compared with that of wild-type OPTN-GFP counterparts ($\boldsymbol{P} < 0.01$). This study highlights a possible role of OPTN in vesicle trafficking

and Golgi integrity. It also provides in-sights into the possible mechanisms why E50K would exhibit a propensity toward the development of glaucoma.

3.846 RalA-exocyst-dependent Recycling Endosome Trafficking Is Required for the Completion of Cytokinesis
Chen, X-W., Inoue, M., Hsu, S. and Saltiel, A.R.
J. Biol. Chem., ***281(50)***, *38609-38616 (2006)*

In eukaryotic cells, recycling endosome-mediated trafficking contributes to the completion of cytokinesis, in a manner under the control of the centrosome. We report that the exocyst complex and its interacting GTPase RalA play a critical role in this polarized trafficking process. RalA resides in the recycling endosome and relocates from the pericentrosomal region to key cytokinetic structures including the cleavage furrow, and later, the abscission site. This event is coupled to the dynamic redistribution of the exocyst proteins. These associate with the centrosome in interphase and concentrate on the central spindle/midbody during cytokinesis. Disruption of RalA-exocyst function leads to cytokinesis failure in late stages, particularly abscission, resembling the cytokinesis defects induced by loss of centrosome function. These data suggest that RalA and the exocyst may regulate vesicle delivery to the centrosome-related abscission site during the terminal stage of cytokinesis, implicating RalA as a critical regulator of cell cycle progression.

3.847 Peroxisomal membrane permeability and solute transfer
Antonenkov, V.D. anfd Hiltunen, J.K.
Biochim. Biophys. Acta, Mol. Cell Res., ***1763*** *(2006)*

The review is dedicated to recent progress in the study of peroxisomal membrane permeability to solutes which has been a matter of debate for more than 40 years. Apparently, the mammalian peroxisomal membrane is freely permeable to small solute molecules owing to the presence of pore-forming channels. However, the membrane forms a permeability barrier for 'bulky' solutes including cofactors (NAD/H, NADP/H, CoA, and acetyl/acyl-CoA esters) and ATP. Therefore, peroxisomes need specific protein transporters to transfer these compounds across the membrane. Recent electrophysiological studies have revealed channel-forming activities in the mammalian peroxisomal membrane. The possible involvement of the channels in the transfer of small metabolites and in the formation of peroxisomal shuttle systems is described.

3.848 Transition of Galactosyltransferase 1 from Trans-Golgi Cisterna to the Trans Golgi Network (and Back) is Signal Mediated
Schaub, B., Berger, B., Berger, E. and Rohrer, J.
Mol. Biol. Cell, ***17*** *(Suppl), abstract 1814 (2006)*

The Golgi apparatus (GA) is the organelle where complex glycan formation takes place. In addition, it is a major sorting site for proteins destined for various sub-cellular compartments or for secretion. Here we investigate β1,4-galactosyltransferase 1 (galT) and α2,6-sialyltransferase 1 (siaT), two *trans*-Golgi glycosyltransferases, with respect to their different pathways in monensin treated cells. Upon addition of monensin galT dissociates from siaT and the GA and accumulates in swollen vesicles derived from the *trans*-Golgi network (TGN), as shown by co-localization with TGN46, a specific TGN marker.
We analyzed various chimeric constructs of galT and siaT by confocal fluorescence microscopy and time lapse video-microscopy as well as Optiprep density gradient fractionation. We show that the first 13 amino acids of the cytoplasmic tail of galT are necessary and sufficient for its localization to swollen vesicles induced by monensin. We also show that the monensin sensitivity resulting from these 13 amino acids can be conferred to siaT, which leads to the rapid accumulation of the galT-siaT chimera in swollen vesicles upon monensin treatment. Based on these data we suggest that cycling between the *trans*-Golgi *cisterna* and the *trans*-Golgi network of galT is signal mediated.

3.849 Characterization of Membrane-associated APC Pools in Motile versus Non-Motile Epithelial Cells
Siemers, K.A., Caro-Gonzales, H.Y., Nelson, W.J. and Barth, A.I.M.
Mol. Biol. Cell, ***17*** *(Suppl.), abstract 299 (2006)*

The adenomatous polyposis coli (APC) protein has an important role in directed cell migration and localizes to the tip of extending membranes in response to growth factor or integrin-mediated signals. Here we used a biochemical approach to analyze the association of APC and its binding partners with

membranes in motile and non-motile epithelial cells. In non-polarized motile Madin Darby Canine kidney (MDCK) epithelial cells, homogenized under conditions that disrupt the microtubule cytoskeleton, the majority of APC and its binding partners Asef and β-catenin were recovered in the membrane fraction whereas tubulin was cytosolic. To further analyze the different cytosolic and membrane-associated APC pool we used a self-forming iodixanol density gradient. APC was enriched in two membrane fractions: a lower density membrane fraction that co-distributes with E-cadherin and β-catenin and a higher density membrane fraction that co-distributes with Asef, IQGAP and Par-3. In polarized non-motile MDCK epithelial cells the majority of APC was enriched in the lower density membrane fraction with E-cadherin. Disruption of cell-cell adhesion causes a shift of APC from this lower density "E-cadherin"-membrane fraction into the higher density "IQGAP/Asef"-membrane fraction. These results indicate that APC complexes that distribute in these higher density fractions may have a role in cell motility.

3.850 Comprehensive Protein Analysis of Naked2-associated Exocytic Vesicles by LC/MS-MS
Cao, Z. et al
*Mol. Biol. Cell, **17**, (Suppl.), abstract 2621 (2006)*

Polarized epithelial cells have developed specialized mechanisms for targeting *trans*Golgi network (TGN)-derived vesicles to the apical or basolateral membrane. We have reported that Naked2, but not Naked1, interacts with Golgi-processed form of TGFα and escorts TGFα-containing exocytic vesicles from the TGN to the basolateral surface of polarized MDCK cells, where the vesicles dock and fuse in a Naked2 myristoylation-dependent manner. In myristoylation-deficient (G2A) Naked2-expressing MDCK cells, Naked2-associated TGFα-containing vesicles are trapped in the cytoplasm and TGFα is unable to reach the plasma membrane. To determine the protein compositions of these Naked2-associated vesicles, we performed a biochemical enrichment followed by flow cytometric purification. Using a 10-40% iodixanol gradient centrifugation, we first isolated G2A Naked2-EGFP-containing vesicle fractions de-enriched in plasma membrane markers as measured by western blotting for Naked2 and markers of subcellular compartments. This pool of vesicles was then subjected to dual flow sorting using GFP and DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) lipid incorporation to achieve 99.3% purity. Proteomic analysis of this flow-sorted population of vesicles by LC/MS-MS has identified 296 proteins using a criterion of 3 or more peptides per protein present in order to be included in the list. This list includes known trafficking proteins as well as new and previously unrecognized potential trafficking proteins. To date, we have validated colocalization of Annexin I and II in both wild-type and G2A Naked2-associated vesicles. To our knowledge, this represents the first example of a comprehensive protein characterization of a subset of exocytic vesicles.

3.851 Trafficking of Shiga toxin/Shiga-like toxin-1 in human glomerular microvascular endothelial cells and human mesangial cells
Warnier, M. et al
*Kidney Int., **70(12)**, 2085-2091 (2006)*

This study has determined the intracellular transport route of Shiga-like toxin (Stx) and the highly related Shiga toxin in human glomerular microvascular endothelial cells (GMVECs) and mesangial cells. In addition, the effect of tumor necrosis factor-α (TNF-α), which contributes to the pathogenesis of hemolytic-uremic syndrome, was evaluated more profound. Establishing the transport route will provide better understanding of the cytotoxic effect of Stx on renal cells. For our studies, we used receptor-binding B-subunit (StxB), which is identical between Shiga toxin and Stx-1. The transport route of StxB was studied by immunofluorescence microscopy and biochemical assays that allow quantitative analysis of retrograde transport from plasma membrane to Golgi apparatus and endoplasmic reticulum (ER). In both cell types, StxB was detergent-resistant membrane associated and followed the retrograde route. TNF-α upregulated Gb3 expression in mesangial cells and GMVECs, without affecting the efficiency of StxB transport to the ER. In conclusion, our study shows that in human GMVECs and mesangial cells, StxB follows the retrograde route to the Golgi apparatus and the ER. TNF-α treatment increases the amount of cell-associated StxB, but not retrograde transport as such, making it likely that the strong TNF-α-induced sensitization of mesangial cells and GMVECs for the toxic action of Stx is not due to a direct effect on the intracellular trafficking of the toxin.

3.852 Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell–cell contact and protein poly(ADP-ribosyl)ation
Yeh, T-Y. et al
*Biochem. J., **399(3)**, 415-425 (2006)*

PARsylation [poly(ADP-ribosyl)ation] of proteins is implicated in the regulation of diverse physiological processes. Tankyrase is a molecular scaffold with this catalytic activity and has been proposed as a regulator of vesicular trafficking on the basis, in part, of its Golgi localization in non-polarized cells. Little is known about tankyrase localization in polarized epithelial cells. Using MDCK (Madin–Darby canine kidney) cells as a model, we found that E-cadherin-mediated intercellular adhesion recruits tankyrase from the cytoplasm to the lateral membrane (including the tight junction), where it stably associates with detergent-insoluble structures. This recruitment is mostly completed within 8 h of calcium-induced formation of cell–cell contact. Conversely, when intercellular adhesion is disrupted by calcium deprivation, tankyrase returns from the lateral membrane to the cytoplasm and becomes more soluble in detergents. The PARsylating activity of tankyrase promotes its dissociation from the lateral membrane as well as its ubiquitination and proteasome-mediated degradation, resulting in an apparent protein half-life of ~2 h. Inhibition of tankyrase autoPARsylation using H_2O_2-induced NAD^+ depletion or PJ34 [*N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N*,*N*-dimethylacetamide hydrochloride] treatment results in tankyrase stabilization and accumulation at the lateral membrane. By contrast, stabilization through proteasome inhibition results in tankyrase accumulation in the cytoplasm. These data suggest that cell–cell contact promotes tankyrase association with the lateral membrane, whereas PARsylating activity promotes translocation to the cytosol, which is followed by ubiquitination and proteasome-mediated degradation. Since the lateral membrane is a sorting station that ensures domain-specific delivery of basolateral membrane proteins, the regulated tankyrase recruitment to this site is consistent with a role in polarized protein targeting in epithelial cells.

3.853 Quantitative proteomic approach to study subcellular localization of membrane proteins
Sadowski, P.G. et al
*Nature Protocols, **1(4)**, 1778-1789 (2006)*

As proteins within cells are spatially organized according to their role, knowledge about protein localization gives insight into protein function. Here, we describe the LOPIT technique (localization of organelle proteins by isotope tagging) developed for the simultaneous and confident determination of the steady-state distribution of hundreds of integral membrane proteins within organelles. The technique uses a partial membrane fractionation strategy in conjunction with quantitative proteomics. Localization of proteins is achieved by measuring their distribution pattern across the density gradient using amine-reactive isotope tagging and comparing these patterns with those of known organelle residents. LOPIT relies on the assumption that proteins belonging to the same organelle will co-fractionate. Multivariate statistical tools are then used to group proteins according to the similarities in their distributions, and hence localization without complete centrifugal separation is achieved. The protocol requires approximately 3 weeks to complete and can be applied in a high-throughput manner to material from many varied sources.

3.854 Physiological Mouse Brain Aβ Levels Are Not Related to the Phosphorylation State of Threonine-668 of Alzheimer's APP
Sano, Y. et al
*PloS, **1**, e51 (2006)*

Background
Amyloid-β peptide species ending at positions 40 and 42 (Aβ40, Aβ42) are generated by the proteolytic processing of the Alzheimer's amyloid precursor protein (APP). Aβ peptides accumulate in the brain early in the course of Alzheimer's disease (AD), especially Aβ42. The cytoplasmic domain of APP regulates intracellular trafficking and metabolism of APP and its carboxyl-terminal fragments (CTFα, CTFβ). The role of protein phosphorylation in general, and that of the phosphorylation state of APP at threonine-668 (Thr^{668}) in particular, has been investigated in detail by several laboratories (including our own). Some investigators have recently proposed that the phosphorylation state of Thr^{668} plays a pivotal role in governing brain Aβ levels, prompting the current study.
Methodology
In order to evaluate whether the phosphorylation state of Thr^{668} controlled brain Aβ levels, we studied the levels and subcellular distributions of holoAPP, sAPPα, sAPPβ, CTFα, CTFβ, Aβ40 and Aβ42 in brains from “knock-in” mice in which a non-phosphorylatable alanyl residue had been substituted at position 668, replacing the threonyl residue present in the wild-type protein.
Conclusions
The levels and subcellular distributions of holoAPP, sAPPα, sAPPβ, CTFα, CTFβ, Aβ40 and Aβ42 in the brains of Thr^{668}Ala mutant mice were identical to those observed in wild-type mice. These results indicate

that, despite speculation to the contrary, the phosphorylation state of APP at Thr^{668} does not play an obvious role in governing the physiological levels of brain Aβ40 or Aβ42 *in vivo*.

3.855 Cellular Prion Protein and Caveolin-1 Interaction in a Neuronal Cell Line Precedes Fyn/Erk 1/2 Signal Transduction
Toni, M. et al
J. Biomed. Biotechnol., ***206***, *1-13 (2006)*

It has been reported that cellular prion protein (PrPc) is enriched in caveolae or caveolae-like domains with caveolin-1 (Cav-1) participating to signal transduction events by Fyn kinase recruitment. By using the Glutathione-S-transferase (GST)-fusion proteins assay, we observed that PrPc strongly interacts in vitro with Cav-1. Thus, we ascertained the PrPc caveolar localization in a hypothalamic neuronal cell line (GN11), by confocal microscopy analysis, flotation on density gradient, and coimmunoprecipitation experiments. Following the anti-PrPc antibody-mediated stimulation of live GN11 cells, we observed that PrPc clustered on plasma membrane domains rich in Cav-1 in which Fyn kinase converged to be activated. After these events, a signaling cascade through p42/44 MAP kinase (Erk 1/2) was triggered, suggesting that following translocations from rafts to caveolae or caveolae-like domains PrPc could interact with Cav-1 and induce signal transduction events.

3.856 Presenilin-Dependent γ-Secretase on Plasma Membrane and Endosomes Is Functionally Distinct
Fukumori, A. et al
Biochemistry, ***45***, *4907-4914 (2006)*

The presenilin (PS)/γ-secretase complex, which contains not only PS but also Aph-1, PEN-2, and nicastrin, mediates proteolysis of the transmembrane domain of β-amyloid protein precursor (βAPP). Intramembrane proteolysis occurs at the interface between the membrane and cytosol (ε-site) and near the middle of the transmembrane domain (γ-site), generating the βAPP intracellular domain (AICD) and Alzheimer disease-associated Aβ, respectively. Both cleavage sites exhibit some diversity. Changes in the precision of γ-cleavage, which potentially results in secretion of pathogenic Aβ42, have been intensively studied, while those of ε-cleavage have not. Although a number of PS-associated factors have been identified, it is unclear whether any of them physiologically regulate the precision of cleavage by PS/γ-secretase. Moreover, there is currently no clear evidence of whether PS/γ-secretase function differs according to the subcellular site. Here, we show that endocytosis affects the precision of PS-dependent ε-cleavage in cell culture. Relative production of longer AICDε49 increases on the plasma membrane, whereas that of shorter AICDε51 increases on endosomes; however, this occurs without a concomitant major change in the precision of cleavage at γ-sites. Moreover, very similar changes in the precision of ε-cleavage are induced by alteration of the pH. Our findings demonstrate that the precision of ε-cleavage by PS/γ-secretase changes depending upon the conditions and the subcellular location. These results suggest that the precision of cleavage by the PS/γ-secretase complex may be physiologically regulated by the subcellular location and conditions.

3.857 Structure and Cholesterol Dynamics of Caveolae/Raft and Nonraft Plasma Membrane Domains
Gallegos, A.M., Storey, S.M., Kier, A.B., Schroeder, F. and Ball, J.M.
Biochemistry, ***45***, *12100-12116 (2006)*

Despite recognition that the plasma membrane (PM) is comprised of lipid raft domains that are key organizing sites of multiple signaling pathways and other cell functions, limited information is available regarding the structure and function in sterol dynamics of these microdomains. To begin to resolve these issues, MDCK membranes were subfractionated by three different techniques to produce (i) detergent-resistant membranes (DRM) and detergent-soluble membranes (DSM), (ii) nondetergent caveolae/rafts (NDCR), and (iii) nondetergent, affinity-purified caveolae/rafts (ACR) and noncaveolae/nonrafts (NR). ACR exhibited the least cross contamination with other PM domains or intracellular membranes, in marked contrast to DRM that contained the highest level of cross contaminants. Spectral properties of dehydroergosterol (DHE), a naturally occurring fluorescent sterol, showed that ACR, NDCR, and NR did not contain crystalline sterol, consistent with the lack of crystalline sterol in PM of intact cells. In contrast, DRM contained significant levels of crystalline sterol. Fluorescence polarization of membrane probes showed that ACR were the least fluid and had the highest transbilayer fluidity gradient, the most liquid ordered phase, and the sterol dynamics most responsive to sterol carrier protein-2 (SCP-2). In contrast,

DRM had structural properties similar to those of NR, anomalous (very fast) spontaneous sterol dynamics, and sterol dynamics that were unresponsive to SCP-2. Differences between the structural and functional properties of DRM and those of the nondetergent preparations (ACR and NDCR) were not due to the presence of detergent. A nondetergent, affinity-purified (ACR) lipid domain fraction isolated from MDCK cells for the first time revealed unique structural (noncrystalline sterol, liquid-ordered, high transbilayer fluidity gradient) and functional (cholesterol dynamics) properties of lipid rafts as compared to nonrafts (NR). In summary, this study showed membrane microdomains (rafts/caveolae) isolated by three different methodologies have unique structural, functional, and organizational characteristics.

3.858 A role for ion channels in glioma cell invasion
McFerrin, M.B. and Sontheimer, H.
Neuron Glia Biol., ***2(1)***, *39-49 (2006)*

Many cells, including neuronal and glial progenitor cells, stem cells and microglial cells, have the capacity to move through the extracellular spaces of the developing and mature brain. This is particularly pronounced in astrocyte-derived tumors, gliomas, which diffusely infiltrate the normal brain. Although a significant body of literature exists regarding signals that are involved in the guidance of cells and their processes, little attention has been paid to cell-shape and cell-volume changes of migratory cells. However, extracellular spaces in the brain are very narrow and represent a major obstacle that requires cells to dynamically regulate their volume. Recent studies in glioma cells show that this involves the secretion of Cl^- and K^+ with water. Pharmacological inhibition of Cl^- channels impairs their ability to migrate and limits tumor progression in experimental tumor models. One Cl^--channel inhibitor, chlorotoxin, is currently in Phase II clinical trials to treat malignant glioma. This article reviews our current knowledge of cell-volume changes and the role of ion channels during the migration of glioma cells. It also discusses evidence that supports the importance of channel-mediated cell-volume changes in the migration of immature neurons and progenitor cells during development. New unpublished data is presented, which demonstrates that Cl^- and K^+ channels involved in cell shrinkage localize to lipid-raft domains on the invadipodia of glioma cells and that their presence might be regulated by trafficking of these proteins in and out of lipid rafts.

3.859 Comparative proteomics of glycosomes from bloodstream form and procyclic culture form *Trypanosoma brucei brucei*
Colasante, C., Ellis, M., Ruppert, T. And Voncken, F.
Proteomics, ***6(11)***, *3275-3293 (2006)*

Peroxisomes are present in nearly every eukaryotic cell and compartmentalize a wide range of important metabolic processes. Glycosomes of Kinetoplastid parasites are peroxisome-like organelles, characterized by the presence of the glycolytic pathway. The two replicating stages of *Trypanosoma brucei brucei*, the mammalian bloodstream form (BSF) and the insect (procyclic) form (PCF), undergo considerable adaptations in metabolism when switching between the two different hosts. These adaptations involve also substantial changes in the proteome of the glycosome. Comparative (non-quantitative) analysis of BSF and PCF glycosomes by nano LC-ESI-Q-TOF-MS resulted in the validation of known functional aspects of glycosomes and the identification of novel glycosomal constituents.

3.860 Proteomic Analysis of Mature Melanosomes from the Retinal Pigmented Epithelium
Azarian, S.M., McLeod, I., Lillo, C., Gibbs, D., Yates, J.R. and Williams, D.S.
J. Proteome Res., ***5(3)***, *521-529 (2006)*

The protein content of melanosomes in the retinal pigment epithelium (RPE) was analyzed by mass spectrometry. More than 100 proteins were found to be common to two out of three variations of sample preparation. Some proteins normally associated with other organelles were detected. Several lysosomal enzymes were detected, with the presence of cathepsin D confirmed by immunoelectron microscopy, thus supporting the previously suggested notion that melanosomes may contribute to the degradation of ingested photoreceptor outer segment disks.

3.861 Insights into the membrane proteome of rat liver peroxisomes: Microsomal glutathione-S-transferase is shared by both subcellular compartments
Islinger, M., Lüers, G.H., Zischka, H., Ueffing, M. and Völkl, A.
Proteomics, ***6(3),*** *804-816 (2006)*

Peroxisomes are ubiquitous “multipurpose” organelles of eukaryotic cells. Their matrix enzymes catalyze mainly catabolic and anabolic reactions of lipid metabolism, thus contributing to the regulation of lipid homeostasis. Since most metabolites must be actively transported across the peroxisomal membrane and since individual proteins and protein complexes play functional roles in such transport processes, we analyzed the peroxisomal membrane proteome. Benzyldimethyl-*n*-hexadecylammoniumchloride (16-BAC)/SDS-2-D-PAGE and mass spectrometry were used to characterize the proteomes of highly purified “light” and “heavy” peroxisomes of rat liver obtained by density gradient centrifugation. In both populations, the major integral membrane proteins could be detected in high concentrations, verifying 16-BAC/SDS-2-D-PAGE as a suitable tool for the preparation of membrane proteomes destined for mass spectrometric analysis. Both reliable and reproducible detection of a distinct set of microsomal (ER) membrane proteins, including microsomal glutathione-S-transferase (mGST), in light and heavy peroxisomal fractions was also possible. Compared with the abundance of most microsomal membrane proteins, we found mGST to be specifically enriched in peroxisomal membrane fractions. Furthermore, C terminus epitope-tagged mGST versions were localized at least in part to peroxisomes in different mammalian cell lines. Taken together, these data suggest that the peroxisomal GST is not a mere ER-contaminant, but a *bona fide* protein comprising the membrane proteome of both intracellular compartments. In addition, we could detect several mitochondrial proteins in light peroxisome fractions. This finding may likely indicate a physical association of light peroxisomes with mitochondria, since the organelles could be partly separated by mechanical stress. Whether this association is of functional importance awaits further investigation.

3.862 Mitochondrial protein patterns correlating with impaired insulin secretion from INS-1E cells exposed to elevated glucose concentrations
Nyblom, H.K., Thorn, K., Ahmed, M. and Bergsten, P.
*Proteomics, **6(19)**, 5193-5198 (2006)*

Extended hyperglycaemia leads to impaired glucose-stimulated insulin secretion (GSIS) and eventually β-cell apoptosis in individuals with type 2 diabetes mellitus. In an attempt to dissect mechanisms behind the detrimental effects of glucose, we focused on measuring changes in expression patterns of mitochondrial proteins. Impaired GSIS was observed from INS-1E cells cultured for 5 days at 20 or 27 mM glucose compared to cells cultured at 5.5 or 11 mM glucose. After culture, mitochondria were isolated from the INS-1E cells by differential centrifugation. Proteins of the mitochondrial fraction were bound to a strong anionic surface (SAX2) protein array and mass spectra generated by SELDI-TOF-MS. Analysis of the spectra revealed proteins with expression levels that correlated with the glucose concentration of the culture medium. Indeed, such differentially expressed proteins created patterns of protein changes, which correlated with impairment of GSIS. In conclusion, the study reveals the first glucose-induced differentially expressed patterns of β-cell mitochondrial proteins obtained by SELDI-TOF-MS.

3.863 Proteomic analysis of detergent-resistant membranes from *Candida albicans*
Insenser, M., Nombela, C., Molero, G. and Gil, C.
*Proteomics, **6, Suppl. 1.,** S74-S81 (2006)*

Lipid rafts are membrane microdomains with a higher amount of saturated fatty acids and sterols than the rest of the membrane. They are more resistant to the action of non-anionic detergents, and are called, for this reason, detergent-resistant membranes (DRMs). Lipid rafts are involved in many cellular processes, like signaling, cytokinesis, response to environment, *etc*., and therefore must contain important proteins. We have obtained a fraction enriched in proteins from *Candida albicans* DRMs. The sample has been analyzed by SDS-PAGE and 29 proteins have been identified including markers for lipid rafts in *Saccharomyces cerevisiae*, like Pma1p and a glycosylphosphatidylinositol (GPI)-anchored protein belonging to the Phr family. Ecm33p, a GPI-anchored protein involved in cell wall biogenesis, has been found for the first time in lipid rafts. We have also identified proteins implicated in protein glycosylation, like the mannosyltransferases Mnn7p, Pmt2p and Mnt1p; proteins involved in lipid metabolism, like Erg11p and Scs7p; and heat shock proteins, like Ssa1p and Hsp90p. Most of the proteins identified are located in plasma, mitochondrial, Golgi or ER membranes, supporting the postulated existence of lipid-raft domains in all the membranes.

3.864 Functionally different pools of Shiga toxin receptor, globotriaosyl ceramide, in HeLa cells
Falguierres, T., Römer, W., Amessou, M., Afonso, C., Wolf, C., Tabet, J-C., Lamaze, C. and Johannes, L.
*FEBS J., **273(22)**, 5205-5218 (2006)*

Many studies have investigated the intracellular trafficking of Shiga toxin, but very little is known about the underlying dynamics of its cellular receptor, the glycosphingolipid globotriaosyl ceramide. In this study, we show that globotriaosyl ceramide is required not only for Shiga toxin binding to cells, but also for its intracellular trafficking. Shiga toxin induces globotriaosyl ceramide recruitment to detergent-resistant membranes, and subsequent internalization of the lipid. The globotriaosyl ceramide pool at the plasma membrane is then replenished from internal stores. Whereas endocytosis is not affected in the recovery condition, retrograde transport of Shiga toxin to the Golgi apparatus and the endoplasmic reticulum is strongly inhibited. This effect is specific, as cholera toxin trafficking on GM_1 and protein biosynthesis are not impaired. The differential behavior of both toxins is also paralleled by the selective loss of Shiga toxin association with detergent-resistant membranes in the recovery condition, and comparison of the molecular species composition of plasma membrane globotriaosyl ceramide indicates subtle changes in favor of unsaturated fatty acids. In conclusion, this study demonstrates the dynamic behavior of globotriaosyl ceramide at the plasma membrane and suggests that globotriaosyl ceramide-specific determinants, possibly its molecular species composition, are selectively required for efficient retrograde sorting on endosomes, but not for endocytosis.

3.865 Isolation and analysis of lipid rafts in cell-cell interactions
Landry, A. and Xavier, r.
*Methods Mol. Biol., **341**, 251-282 (2006)*

Lipid rafts are dynamic structures made up of proteins and lipids that float freely within the liquid-disordered bilayer of cellular membranes and have the ability to cluster to form larger, more-ordered platforms. These clustered structures have been identified in all cell types and have been shown to play critical roles in signal transduction, cellular transport, and cell-cell communication. Lipid rafts also have been implicated in facilitating bacterial/viral entry into host cells and in human disease, highlighting the significance of understanding the role lipid rafts play in physiological and pathological signaling outcomes. In this chapter, we provide protocols to isolate lipid rafts from polarized and nonpolarized cells and outline novel technologies to analyze signal transduction cascades in vivo.

3.866 Cardiac mitochondrial connexin 43 regulates apoptosis
Goubaeva, F. et al
*Biochem. Biophys. Res. Comm., **352(1)**, 97-103 (2007)*

Connexin 43 (Cx43) is thought to be present largely in the plasma membrane and its function solely to provide low resistance electrical connection between myocytes. A recent report suggested the presence of Cx43 in the mitochondria as well. We confirmed the presence of Cx43 in the mitochondria isolated from adult rat ventricles with the Cx43 immunoreactivity fractionating to the outer mitochondrial membrane. Mitochondrial Cx43 is mostly phosphorylated only detected by a phospho-specific antibody. Using a Ca^{2+}-sensitive electrode and Western blot, we showed that the gap junction inhibitors 18-β-glycyrrhetinic acid (β-GA), oleamide, and heptanol all induced concomitant release of Ca^{2+} and cytochrome *C* in isolated mitochondria whereas the inactive analog 18-β-glycyrrhizic acid failed to do so. In low density neonatal myocyte culture with no appreciable cell–cell contacts, β-GA induced apoptosis as assessed by TUNEL staining. Our results suggest a novel role of Cx43 as a regulator of mitochondrial physiology and myocyte apoptosis.

3.867 Signaling proteins in raft-like microdomains are essential for Ca2+ wave propagation in glial cells
Weerth, S.H., Holtzclaw, L.A. and Russell, J.T.
*Cell Calsium, **41(1)**, 155-167 (2007)*

The hypothesis that calcium signaling proteins segregate into lipid raft-like microdomains was tested in isolated membranes of rat oligodendrocyte progenitor (OP) cells and astrocytes using Triton X-100 solubilization and density gradient centrifugation. Western blot analysis of gradient fractions showed co-localization of caveolin-1 with proteins involved in the Ca^{2+} signaling cascade. These included agonist receptors, $P2Y_1$, and M_1, TRPC1, IP_3R2, ryanodine receptor, as well as the G protein $G\alpha_q$ and Homer. Membranes isolated from agonist-stimulated astrocytes showed an enhanced recruitment of phospholipase C (PLCβ1), IP_3R2 and protein kinase C (PKC-α) into lipid raft fractions. IP_3R2, TRPC1 and Homer co-immunoprecipitated, suggesting protein–protein interactions. Disruption of rafts by cholesterol depletion using methyl-β-cyclodextrin (β-MCD) altered the distribution of caveolin-1 and GM1 to non-raft fractions with higher densities. β-MCD-induced disruption of rafts inhibited agonist-evoked Ca^{2+} wave propagation

in astrocytes and attenuated wave speeds. These results indicate that in glial cells, Ca^{2+} signaling proteins might exist in organized membrane microdomains, and these complexes may include proteins from different cellular membrane systems. Such an organization is essential for Ca^{2+} wave propagation.

3.868 Characterisation of lipofuscin-like lysosomal inclusion bodies from human placenta
Schröder, B., Elsässer, H-P-. Schmidt, B. and Hasilik, A.
FEBS Lett., ***581(1)***, *102-108 (2007)*

A structural hallmark of lysosomes is heterogeneity of their contents. We describe a method for isolation of particulate materials from human placental lysosomes. After a methionine methyl ester-induced disruption of lysosomes and two density gradient centrifugations we obtained a homogeneous membrane fraction and another one enriched in particulate inclusions. The latter exhibited a yellow-brown coloration and contained bodies lacking a delimiting membrane, which were characterised by a granular pattern and high electron density. The lipofuscin-like inclusion materials were rich in tripeptidyl peptidase I, β-glucuronidase, acid ceramidase and apolipoprotein D and contained proteins originating from diverse subcellular localisations.
Here we show that human term placenta contains lipofuscin-like lysosomal inclusions, a phenomenon usually associated with senescence in postmitotic cells. These findings imply that a simple pelleting of a lysosomal lysate is not appropriate for the isolation of lysosomal membranes, as the inclusions tend to be sedimented with the membranes.

3.869 Activation-induced endocytosis of the raft-associated transmembrane adaptor protein LAB/NTAL in B lymphocytes: evidence for a role in internalization of the B cell receptor
Mutch, C.M. et al
Int. Immunol., ***19(1)***, *19-30 (2007)*

Linker for activation of B cell (LAB)/non-T cell activation linker (NTAL) and phosphoprotein associated with glycophospholipid-enriched membrane microdomain (PAG)/Csk-binding protein (Cbp) are raft-associated transmembrane adaptor proteins with distinct functions in immediate/early phases of receptor signaling pathways. Heterogeneous rafts are thought to compartmentalize membrane-associated signaling events. In order to investigate the subcellular localization of LAB/NTAL and PAG/Cbp, they were expressed as fluorescent chimeric fusion proteins in a human B cell line and their distribution was examined, along with the corresponding endogenous proteins, before and after B cell receptor (BCR) stimulation. Both adaptors were distributed predominantly at the plasma membrane in resting cells and co-clustered with other raft-associated proteins; however, they distributed differently in buoyant membranes isolated by either detergent resistance or non-detergent methods, indicating that they might localize to distinct rafts. After activation, LAB/NTAL was internalized and co-localized with the BCR while PAG/Cbp remained on the cell surface. BCR internalization was reduced in LAB/NTAL-deficient murine B cells, suggesting a regulatory role for LAB/NTAL in activation-induced internalization of the BCR. The cytoplasmic domain of LAB/NTAL, and not the transmembrane/juxtamembrane region, was found to be essential for its internalization.

3.870 Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response
McBroom, A.J. and Kuehn, M.J.
Mol. Microbiol., ***63(2)***, *545-558 (2007)*

Conditions that impair protein folding in the Gram-negative bacterial envelope cause stress. The destabilizing effects of stress in this compartment are recognized and countered by a number of signal transduction mechanisms. Data presented here reveal another facet of the complex bacterial stress response, release of outer membrane vesicles. Native vesicles are composed of outer membrane and periplasmic material, and they are released from the bacterial surface without loss of membrane integrity. Here we demonstrate that the quantity of vesicle release correlates directly with the level of protein accumulation in the cell envelope. Accumulation of material occurs under stress, and is exacerbated upon impairment of the normal housekeeping and stress-responsive mechanisms of the cell. Mutations that cause increased vesiculation enhance bacterial survival upon challenge with stressing agents or accumulation of toxic misfolded proteins. Preferential packaging of a misfolded protein mimic into vesicles for removal indicates that the vesiculation process can act to selectively eliminate unwanted material. Our results demonstrate that production of bacterial outer membrane vesicles is a fully independent, general envelope stress

response. In addition to identifying a novel mechanism for alleviating stress, this work provides physiological relevance for vesicle production as a protective mechanism.

3.871 Down-regulation of dopamine transporter by iron chelation *in vitro* is mediated by altered trafficking, not synthesis
Wiesinger, J.A.
J. Neurochem., ***100***, *167-179 (2007)*

Neurological development and functioning of dopamine (DA) neurotransmission is adversely affected by iron deficiency in early life. Iron-deficient rats demonstrate significant elevations in extracellular DA and a reduction in dopamine transporter (DAT) densities in the caudate putamen and nucleus accumbens. To explore possible mechanisms by which cellular iron concentrations control DAT functioning, endogenous DAT-expressing PC12 cells were used to determine the effect of iron chelation on DAT protein and mRNA expression patterns. In addition, we used human DAT (hDAT)-transfected Neuro2a (N2A) cells to examine DAT degradation and trafficking patterns. A 50 μ**m** treatment for 24 h with the iron chelator, desferrioxamine (DFO), significantly decreased dopamine uptake in a dose-dependent manner, with no apparent change in K_m, in both PC12 and N2A cells. Reduced DA uptake was accompanied by concentration- and time-dependent reductions in total DAT protein levels in both cell lines. Exposure to increasing concentrations of DFO did not significantly alter DAT mRNA in either PC12 or N2A cells. However, DAT degradation rates increased three–fivefold in both cell types exposed to 50 μ**m** DFO for 24 h. Biotinylation studies in N2A cells indicate a more dramatic loss of DAT in the membrane fraction, while **OptiPrep** fractionation experiments revealed an increase in lysosomal DAT with iron chelation. Inhibition of protein kinase C activation with staurosporin prevented the effect of iron chelation on DAT function, suggesting that *in vitro* iron chelation affects DAT primarily through the effects on trafficking rather than on synthesis.

3.872 Inhibition of the Secretory Pathway by Foot-and-Mouth Disease Virus 2BC Protein Is Reproduced by Coexpression of 2B with 2C, and the Site of Inhibition Is Determined by the Subcellular Location of 2C
Moffat, K. et al
J. Virol., ***81(3)***, *1129-1139 (2007)*

Infection of cells with picornaviruses can lead to a block in protein secretion. For poliovirus this is achieved by the 3A protein, and the consequent reduction in secretion of proinflammatory cytokines and surface expression of major histocompatibility complex class I proteins may inhibit host immune responses in vivo. Foot-and-mouth disease virus (FMDV), another picornavirus, can cause persistent infection of ruminants, suggesting it too may inhibit immune responses. Endoplasmic reticulum (ER)-to-Golgi apparatus transport of proteins is blocked by the FMDV 2BC protein. The observation that 2BC is processed to 2B and 2C during infection and that individual 2B and 2C proteins are unable to block secretion stimulated us to study the effects of 2BC processing on the secretory pathway. Even though 2BC was processed rapidly to 2B and 2C, protein transport to the plasma membrane was still blocked in FMDV-infected cells. The block could be reconstituted by coexpression of 2B and 2C, showing that processing of 2BC did not compromise the ability of FMDV to slow secretion. Under these conditions, 2C was located to the Golgi apparatus, and the block in transport also occurred in the Golgi apparatus. Interestingly, the block in transport could be redirected to the ER when 2B was coexpressed with a 2C protein fused to an ER retention element. Thus, for FMDV a block in secretion is dependent on both 2B and 2C, with the latter determining the site of the block.

3.873 Membrane Cholesterol Content Modulates ClC-2 Gating and Sensitivity to Oxidative Stress
Hinzpeter, A. et al
J. Biol. Chem., ***282(4)***, *2423-2432 (2007)*

ClC-2 is a broadly expressed member of the voltage-gated ClC chloride channel family. In this study, we aimed to evaluate the role of the membrane lipid environment in ClC-2 function, and in particular the effect of cholesterol and ClC-2 distribution in membrane microdomains. Detergent-resistant and detergent-soluble microdomains (DSM) were isolated from stably transfected HEK293 cells by a discontinuous **OptiPrep** gradient. ClC-2 was found concentrated in detergent-insoluble membranes in basal conditions and relocalized to DSM upon cholesterol depletion by methyl-β-cyclodextrin. As assessed by patch clamp recordings, relocalization was accompanied by acceleration of the activation kinetics of the channel. A

similar distribution and activation pattern were obtained when cells were treated with the oxidant *tert*-butyl hydroperoxide and after ATP depletion. In both cases activation was prevented by cholesterol enrichment of cells. We conclude that the cholesterol environment regulates ClC-2 activity, and we provide evidence that the increase in ClC-2 activity in response to acute oxidative or metabolic stress involves relocalization of this channel to DSM.

3.874 Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts
Mammoto, A., Huang, S. and Ingber, D.E.
*J. Cell Sci., **120**, 456-467 (2007)*

Cytoskeleton-dependent changes in the activity of the small GTPase Rho mediate the effects of cell shape on cell function; however, little is known about how cell spreading and related distortion of the cytoskeleton regulate Rho activity. Here we show that rearrangements of the actin cytoskeleton associated with early phases of cell spreading in human microvascular endothelial (HMVE) cells suppress Rho activity by promoting accumulation of p190RhoGAP in lipid rafts where it exerts its Rho inhibitory activity. p190RhoGAP is excluded from lipid rafts and Rho activity increases when cell rounding is induced or the actin cytoskeleton is disrupted, and p190RhoGAP knockdown using siRNA prevents Rho inactivation by cell spreading. Importantly, cell rounding fails to prevent accumulation of p190RhoGAP in lipid rafts and to increase Rho activity in cells that lack the cytoskeletal protein filamin. Moreover, filamin is degraded in spread cells and cells that express a calpain-resistant form of filamin exhibit high Rho activity even when spread. Filamin may therefore represent the missing link that connects cytoskeleton-dependent changes of cell shape to Rho inactivation during the earliest phases of cell spreading by virtue of its ability to promote accumulation of p190RhoGAP in lipid rafts.

3.875 Lethal recessive myelin toxicity of prion protein lacking its central domain
Baumann, F. et al
*EMBO J., **26**, 538-547 (2007)*

PrP^C-deficient mice expressing prion protein variants with large amino-proximal deletions (termed $PrP\Delta_F$) suffer from neurodegeneration, which is rescued by full-length PrP^C. We now report that expression of PrP Δ_{CD}, a PrP variant lacking 40 central residues (94–134), induces a rapidly progressive, lethal phenotype with extensive central and peripheral myelin degeneration. This phenotype was rescued dose-dependently by coexpression of full-length PrP^C or PrP^C lacking all octarepeats. Expression of a PrP^C variant lacking eight residues (114–121) was innocuous in the presence or absence of full-length PrP^C, yet enhanced the toxicity of $PrP\Delta_{CD}$ and diminished that of $PrP\Delta_F$. Therefore, deletion of the entire central domain generates a strong recessive-negative mutant of PrP^C, whereas removal of residues 114–121 creates a partial agonist with context-dependent action. These findings suggest that myelin integrity is maintained by a constitutively active neurotrophic protein complex involving PrP^C, whose effector domain encompasses residues 94–134.

3.876 c-Jun Downregulation by HDAC3-Dependent Transcriptional Repression Promotes Osmotic Stress-Induced Cell Apoptosis
Xia, Y. et al
*Mol. Cell, **25**, 219-232 (2007)*

c-Jun, a major transcription factor in the activating protein 1 (AP-1) family of regulatory proteins, is activated by many physiologic and pathologic stimuli. However, whether c-*jun* is regulated by epigenetic modification of chromatin structure is not clear. We showed here that c-*jun* was transcriptionally repressed in response to osmotic stress via a truncated HDAC3 generated by caspase-7-dependent cleavage at aspartic acid 391. The activation of caspase-7, which is independent of cytochrome *c* release and activation of caspase-9 and caspase-12, depends on activation of caspase-8, which in turn requires MEK2 activity and secretion of FAS ligand. The cell apoptosis induced by the truncated HDAC3 or enhanced by c-Jun deficiency during osmotic stress was suppressed by exogenous expression of c-Jun, indicating that the downregulation of c-Jun by HDAC3-dependent transcriptional repression plays a role in regulating cell survival and apoptosis.

3.877 Heterogeneity of Raft-Type Membrane Microdomains Associated with VP4, the Rotavirus Spike Protein, in Caco-2 and MA 104 Cells
Delmas, O. Et al

J. Virol., ***81(4)***, *1610-1618 (2007)*

Previous studies have shown that rotavirus virions, a major cause of infantile diarrhea, assemble within small intestinal enterocytes and are released at the apical pole without significant cell lysis. In contrast, for the poorly differentiated kidney epithelial MA 104 cells, which have been used extensively to study rotavirus assembly, it has been shown that rotavirus is released by cell lysis. The subsequent discovery that rotavirus particles associate with raft-type membrane microdomains (RTM) in Caco-2 cells provided a simple explanation for rotavirus polarized targeting. However, the results presented here, together with those recently published by another group, demonstrate that rotavirus also associates with RTM in MA 104 cells, thus indicating that a simple interaction of rotavirus with rafts is not sufficient to explain its apical targeting in intestinal cells. In the present study, we explore the possibility that RTM may have distinct physicochemical properties that may account for the differences observed in the rotavirus cell cycle between MA 104 and Caco-2 cells. We show here that VP4 association with rafts is sensitive to cholesterol extraction by methyl-ß-cyclodextrin treatment in MA 104 cells and insensitive in Caco-2 cells. Using the VP4 spike protein as bait, VP4-enriched raft subsets were immunopurified. They contained 10 to 15% of the lipids present in total raft membranes. We found that the nature and proportion of phospholipids and glycosphingolipids were different between the two cell lines. We propose that this raft heterogeneity may support the cell type dependency of virus assembly and release.

3.878 **Identification of Differentially Activated Cell-Signaling Networks Associated with Pichinde Virus Pathogenesis by Using Systems Kinomics**
Bowick, G.C. et al
J. Virol., ***81(4)***, *1923-1933 (2007)*

Phosphorylation plays a key role in regulating many signaling pathways. Although studies investigating the phosphorylated forms of signaling pathways are now commonplace, global analysis of protein phosphorylation and kinase activity has lagged behind genomics and proteomics. We have used a kinomics approach to study the effect of virus infection on host cell signaling in infected guinea pigs. Delineating the host responses which lead to clearance of a pathogen requires the use of a matched, comparative model system. We have used two passage variants of the arenavirus Pichinde, used as a biosafety level 2 model of Lassa fever virus as it produces similar pathologies in guinea pigs and humans, to compare the host cell responses between infections which lead to either a mild, self-limiting infection or lethal disease. Using this model, we can begin to understand the differences in signaling events which give rise to these markedly different outcomes. By contextualizing these data using pathway analysis, we have identified key differences in cellular signaling matrices. By comparing these differentially involved networks, we have identified a number of key signaling "nodes" which show differential phosphorylations between mild and lethal infections. We believe that these nodes provide potential targets for the development of antiviral therapies by acting at the level of the host response rather than by directly targeting viral proteins.

3.879 **The reduced GM-CSF priming of ROS production in granulocytes from patients with myelodysplasia is associated with an impaired lipid raft formation**
Fuhler, G.M., Blom, N.R., Coffer, P.J., Drayer, A.L. and Vellenga, E.
J. Leukoc. Biol., ***81***, *449-457 (2007)*

Patients with myelodysplasia (MDS) show an impaired reactive oxygen species (ROS) production in response to fMLP stimulation of GM-CSF-primed neutrophils. In this study, we investigated the involvement of lipid rafts in this process and showed that treatment of neutrophils with the lipid raft-disrupting agent methyl-ß-cyclodextrin abrogates fMLP-induced ROS production and activation of ERK1/2 and protein kinase B/Akt, two signal transduction pathways involved in ROS production in unprimed and GM-CSF-primed neutrophils. We subsequently showed that there was a decreased presence of Lyn, $gp91^{phox}$, and $p22^{phox}$ in lipid raft fractions from neutrophils of MDS. Furthermore, the plasma membrane expression of the lipid raft marker GM1, which increases upon stimulation of GM-CSF-primed cells with fMLP, was reduced significantly in MDS patients. By electron microscopy, we showed that the fMLP-induced increase in GM1 expression in GM-CSF-primed cells was a result of de novo synthesis, which was less efficient in MDS neutrophils. Taken together, these data indicate an involvement of lipid rafts in activation of signal transduction pathways leading to ROS production and show that in MDS neutrophils, an impaired lipid raft formation in GM-CSF-primed cells results in an impaired ROS production.

3.880 Novel peroxisomal protease Tysnd1 processes PTS1- and PTS2-containing enzymes involved in β-oxidation of fatty acids
Kurochkin, I.V. et al
*EMBO J., **26**, 835-845 (2007)*

Peroxisomes play an important role in β-oxidation of fatty acids. All peroxisomal matrix proteins are synthesized in the cytosol and post-translationally sorted to the organelle. Two distinct peroxisomal signal targeting sequences (PTSs), the C-terminal PTS1 and the N-terminal PTS2, have been defined. Import of precursor PTS2 proteins into the peroxisomes is accompanied by a proteolytic removal of the N-terminal targeting sequence. Although the PTS1 signal is preserved upon translocation, many PTS1 proteins undergo a highly selective and limited cleavage. Here, we demonstrate that Tysnd1, a previously uncharacterized protein, is responsible both for the removal of the leader peptide from PTS2 proteins and for the specific processing of PTS1 proteins. All of the identified Tysnd1 substrates catalyze peroxisomal β-oxidation. Tysnd1 itself undergoes processing through the removal of the presumably inhibitory N-terminal fragment. Tysnd1 expression is induced by the proliferator-activated receptor αagonist bezafibrate, along with the increase in its substrates. A model is proposed where the Tysnd1-mediated processing of the peroxisomal enzymes promotes their assembly into a supramolecular complex to enhance the rate of β-oxidation.

3.881 Relationships between the Sequence of α-Synuclein and its Membrane Affinity, Fibrillization Propensity, and Yeast Toxicity
Volles, M.J. and Lansbury, P.T.
*J. Mol. Biol., **366(5)**, 1510-1522 (2007)*

To investigate the α-synuclein protein and its role in Parkinson's disease, we screened a library of random point mutants both *in vitro* and in yeast to find variants in an unbiased way that could help us understand the sequence–phenotype relationship. We developed a rapid purification method that allowed us to screen 59 synuclein mutants *in vitro* and discovered two double-point mutants that fibrillized slowly relative to wild-type, A30P, and A53T α-synucleins. The yeast toxicity of all of these proteins was measured, and we found no correlation with fibrillization rate, suggesting that fibrillization is not necessary for synuclein-induced yeast toxicity. We found that β-synuclein was of intermediate toxicity to yeast, and γ-synuclein was non-toxic. Co-expression of Parkinson's disease-related genes DJ-1, parkin, Pink1, UCH-L1, or synphilin, with synuclein, did not affect synuclein toxicity. A second screen, of several thousand library clones in yeast, identified 25 non-toxic α-synuclein sequence variants. Most of these contained a mutation to either proline or glutamic acid that caused a defect in membrane binding. We hypothesize that yeast toxicity is caused by synuclein binding directly to membranes at levels sufficient to non-specifically disrupt homeostasis.

3.882 Asymmetric Localization of Calpain 2 during Neutrophil Chemotaxis
Nuzzi, P.A., Senetar, M.A., Huttenlocher, A.
*Mol. Biol. Cell, **18**, 795-805 (2007)*

Chemoattractants induce neutrophil polarization through localized polymerization of F-actin at the leading edge. The suppression of rear and lateral protrusions is required for efficient chemotaxis and involves the temporal and spatial segregation of signaling molecules. We have previously shown that the intracellular calcium-dependent protease calpain is required for cell migration and is involved in regulating neutrophil chemotaxis. Here, we show that primary neutrophils and neutrophil-like HL-60 cells express both calpain 1 and calpain 2 and that chemoattractants induce the asymmetric recruitment of calpain 2, but not calpain 1, to the leading edge of polarized neutrophils and differentiated HL-60 cells. Using time-lapse microscopy, we show that enrichment of calpain 2 at the leading edge occurs during early pseudopod formation and that its localization is sensitive to changes in the chemotactic gradient. We demonstrate that calpain 2 is recruited to lipid rafts and that cholesterol depletion perturbs calpain 2 localization, suggesting that its enrichment at the front requires proper membrane organization. Finally, we show that catalytic activity of calpain is required to limit pseudopod formation in the direction of chemoattractant and for efficient chemotaxis. Together, our findings identify calpain 2 as a novel component of the frontness signal that promotes polarization during chemotaxis.

3.883 PALS1 Regulates E-Cadherin Trafficking in Mammalian Epithelial Cells
Wang, Q., Chen, X-W. and Margolis, B.
*Mol. Biol. Cell, **18**, 874-885 (2007)*

Protein Associated with *Lin Seven 1* (PALS1) is an evolutionarily conserved scaffold protein that targets to the tight junction in mammalian epithelia. Prior work in our laboratory demonstrated that the knockdown of PALS1 in Madin Darby canine kidney cells leads to tight junction and polarity defects. We have created new PALS1 stable knockdown cell lines with more profound reduction of PALS1 expression, and a more severe defect in tight junction formation was observed. Unexpectedly, we also observed a severe adherens junction defect, and both defects were corrected when PALS1 wild type and certain PALS1 mutants were expressed in the knockdown cells. We found that the adherens junction structural component E-cadherin was not effectively delivered to the cell surface in the PALS1 knockdown cells, and E-cadherin puncta accumulated in the cell periphery. The exocyst complex was also found to be mislocalized in PALS1 knockdown cells, potentially explaining why E-cadherin trafficking is disrupted. Our results suggest a broad and evolutionarily conserved role for the tight junction protein PALS1 in the biogenesis of adherens junction.

3.884 Role of the Sec61 Translocon in EGF Receptor Trafficking to the Nucleus and Gene Expression
Liao, H-J. and Carpenter, G.
*Mol. Biol. Cell, **18**, 1064-1072 (2007)*

The epidermal growth factor (EGF)-dependent trafficking of the intact EGF receptor to the nucleus and its requirement for growth factor induction of cyclin D and other genes has been reported. Unresolved is the mechanism by which this or other transmembrane proteins are excised from a lipid bilayer before nuclear translocalization. We report that, after the addition of EGF, the cell surface EGF receptor is trafficked to the endoplasmic reticulum (ER) where it associates with Sec61β, a component of the Sec61 translocon, and is retrotranslocated from the ER to the cytoplasm. Abrogation of Sec61β expression prevents EGF-dependent localization of EGF receptors to the nucleus and expression of cyclin D. This indicates that EGF receptors are trafficked from the ER to the nucleus by a novel pathway that involves the Sec61 translocon.

3.885 Conservation of the TRAPPII-specific subunits of a Ypt/Rab exchanger complex
Cox, R., Chen, S.H., Yoo, E. and Segev, N.
*BMC Evolutionary Biol., **7(12)**, 1-15 (2007)*

Background
Ypt/Rab GTPases and their GEF activators regulate intra-cellular trafficking in all eukaryotic cells. In *S. cerivisiae*, the modular TRAPP complex acts as a GEF for the Golgi gatekeepers: Ypt1 and the functional pair Ypt31/32. While TRAPPI, which acts in early Golgi, is conserved from fungi to animals, not much is known about TRAPPII, which acts in late Golgi and consists of TRAPPI plus three additional subunits.
Results
Here, we show a phylogenetic analysis of the three TRAPPII-specific subunits. One copy of each of the two essential subunits, Trs120 and Trs130, is present in almost every fully sequenced eukaryotic genome. Moreover, the primary, as well as the predicted secondary, structure of the Trs120- and Trs130-related sequences are conserved from fungi to animals. The mammalian orthologs of Trs120 and Trs130, NIBP and TMEM1, respectively, are candidates for human disorders. Currently, NIBP is implicated in signaling, and TMEM1 is suggested to have trans-membrane domains (TMDs) and to function as a membrane channel. However, we show here that the yeast Trs130 does not function as a trans-membrane protein, and the human TMEM1 does not contain putative TMDs. The non-essential subunit, Trs65, is conserved only among many fungi and some unicellular eukaryotes. Multiple alignment analysis of each TRAPPII-specific subunit revealed conserved domains that include highly conserved amino acids.
Conclusion
We suggest that the function of both NIBP and TMEM1 in the regulation of intra-cellular trafficking is conserved from yeast to man. The conserved domains and amino acids discovered here can be used for functional analysis that should help to resolve the differences in the assigned functions of these proteins in fungi and animals.

3.886 Transcriptome analysis reveals the population of dendritic RNAs and their redistribution by neural activity
Matsumoto, M., Setou, M. and Inokuchi, K.
*Neurosci. Res., **57**, 411-423 (2007)*

Subcellular localization of RNA is an efficient way to localize proteins to a specific region of a cell. The dendritic localization of RNAs underlies the establishment and maintenance of the synaptic functions of neuronal cells. A requirement for dendritic RNA localization and subsequent local translation has been demonstrated in several forms of experience-dependent synaptic plasticity. In spite of several attempts to identify these RNAs, the population of RNA species present in dendrites as a whole has not been well described. Here we show the results of microarray analyses with RNAs isolated from heavy portion of polysome (HP) fraction where RNA granules are enriched in and synaptosome fraction, prepared from the rat brain. These analyses revealed the complex nature of the dendritic RNA population, which included RNAs that were not expected to be in the dendrites. Neural activity caused by an electroconvulsive shock triggered a redistribution of the population of dendritic transcriptome towards the area of overlap between the HP and the synaptosome, which is assumed to be neck of spine. This redistribution may accompany some changes in the translatability of those transcriptome, which suggests complex mechanisms of local translation in response to synaptic inputs.

3.887 Retinoschisin Is a Peripheral Membrane Protein with Affinity for Anionic Phospholipids and Affected by Divalent Cations

Vijayasarathy, C., Takada, Y., Zeng, Y., Bush, R.A. and Sieving, P.A.
Invest. Ophtalmol. Vis. Sci., ***48****, 991-1000 (2007)*

PURPOSE. Retinoschisin (RS) is a retina-specific, secreted protein implicated in X-linked juvenile retinoschisis and essential for the structural and functional integrity of the retina. This biochemical characterization and ultrastructural localization of RS in intact murine retina was performed to further understanding of the molecular basis of its function.
METHODS. Subcellular fractions and fractions enriched in photoreceptor inner and outer segments were prepared from mouse retina by differential or density gradient ultracentrifugation. Immunoblot analysis was used to assess the expression of RS in various subcellular compartments and its fractionation into soluble phase on treatment of retinal cell membranes with several solubilizing reagents. RS–lipid interactions were evaluated by a protein–lipid overlay assay that used wild-type and mutant forms of RS discoidin domain glutathione S-transferase (GST) fusion proteins. The subcellular localization of RS in mouse retina was visualized by pre-embedding immunogold electron microscopy. Ultrastructure was evaluated by transmission electron microscopy.
RESULTS. RS was intimately associated with cell membranes of the retina. It was found to cluster on the outer leaflet of the plasma membrane of the photoreceptor inner segments, which synthesize and secrete it. It was released from the membrane at high pH, which is characteristic of a peripheral membrane protein. It was extracted from the membrane by the nonionic detergent NP-40, together with glycerophospholipids. Protein–lipid overlay assays indicated a preferential interaction between RS and anioic phospholipids. Extraction of RS from the membrane was inhibited by divalent cations. Photoreceptor inner segment morphology was markedly affected in $RS^{-/y}$ mice, which failed to express RS protein.
CONCLUSIONS. RS in intact retina is a peripheral membrane protein. Although distributed over the two membrane faces, RS is associated primarily with the outer leaflet of the inner segment plasma membrane through anionic phospholipids and divalent cations. RS's localization in photoreceptors and its biochemical properties suggest a functional role locally, at the site of secretion and membrane adhesion, in maintaining the photoreceptor inner segment stability and architecture.

3.888 Insulin-stimulated exocytosis of GLUT4 is enhanced by IRAP and its partner tankyrase

Yeh, T-Y.J., Sbodio, J.I., Tsun, Z-Y., Luo, B. and Chi, N-W.
Biochem. J., ***402****, 279-290 (2007)*

The glucose transporter GLUT4 and the aminopeptidase IRAP (insulin-responsive aminopeptidase) are the major cargo proteins of GSVs (GLUT4 storage vesicles) in adipocytes and myocytes. In the basal state, most GSVs are sequestered in perinuclear and other cytosolic compartments. Following insulin stimulation, GSVs undergo exocytic translocation to insert GLUT4 and IRAP into the plasma membrane. The mechanisms regulating GSV trafficking are not fully defined. In the present study, using 3T3-L1 adipocytes transfected with siRNAs (small interfering RNAs), we show that insulin-stimulated IRAP translocation remained intact despite substantial GLUT4 knockdown. By contrast, insulin-stimulated GLUT4 translocation was impaired upon IRAP knockdown, indicating that IRAP plays a role in GSV trafficking. We also show that knockdown of tankyrase, a Golgi-associated IRAP-binding protein that co-localizes with perinuclear GSVs, attenuated insulin-stimulated GSV translocation and glucose uptake without disrupting insulin-induced phosphorylation cascades. Moreover, iodixanol density gradient

analyses revealed that tankyrase knockdown altered the basal-state partitioning of GLUT4 and IRAP within endosomal compartments, apparently by shifting both proteins toward less buoyant compartments. Importantly, the afore-mentioned effects of tankyrase knockdown were reproduced by treating adipocytes with PJ34, a general PARP (poly-ADP-ribose polymerase) inhibitor that abrogated tankyrase-mediated protein modification known as poly-ADP-ribosylation. Collectively, these findings suggest that physiological GSV trafficking depends in part on the presence of IRAP in these vesicles, and that this process is regulated by tankyrase and probably its PARP activity.

3.889 **p56lck, LFA-1 and PI3K but not SHP-2 interact with GM1- or GM3-enriched microdomains in a CD4–p56lck association-dependent manner**
Barbat, C. et al
Biochem. J., ***402(3)***, *471-481 (2007)*

We previously showed that the association of CD4 and G_{M3} ganglioside induced by CD4 ligand binding was required for the down-regulation of adhesion and that aggregation of ganglioside-enriched domains was accompanied by transient co-localization of LFA-1 (lymphocyte function-associated antigen-1), PI3K (phosphoinositide 3-kinase) and CD4. We also showed that these proteins co-localized with the G_{M1} ganglioside that partially co-localized with G_{M3} in these domains. In the present study, we show that CD4–$p56^{lck}$ association in CD4 signalling is required for the redistribution of $p56^{lck}$, PI3K and LFA-1 in ganglioside-enriched domains, since ganglioside aggregation and recruitment of these proteins were not observed in a T-cell line (A201) expressing the mutant form of CD4 that does not bind $p56^{lck}$. In addition, we show that although these proteins associated in different ways with G_{M1} and G_{M3}, all of the associations were dependent on CD4–$p56^{lck}$ association. Gangliosides could associate with these proteins that differ in affinity binding and could be modified following CD4 signalling. Our results suggest that through these associations, gangliosides transiently sequestrate these proteins and consequently inhibit LFA-1-dependent adhesion. Furthermore, while structural diversity of gangliosides may allow association with distinct proteins, we show that the tyrosine phosphatase SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2), also required for the down-regulation of LFA-1-dependent adhesion, transiently and partially co-localized with PI3K and $p56^{lck}$ in detergent-insoluble membranes without association with G_{M1} or G_{M3}. We propose that CD4 ligation and binding with $p56^{lck}$ and their interaction with G_{M3} and/or G_{M1} gangliosides induce recruitment of distinct proteins important for CD4 signalling to form a multimolecular signalling complex.

3.890 **EDEM1 reveals a quality control vesicular transport pathway out of the endoplasmic reticulum not involving the COPII exit sites**
Zuber, C. et al
PNAS, ***104(11)***, *4407-4412 (2007)*

Immature and nonnative proteins are retained in the endoplasmic reticulum (ER) by the quality control machinery. Folding-incompetent glycoproteins are eventually targeted for ER-associated protein degradation (ERAD). EDEM1 (ER degradation-enhancing α-mannosidase-like protein 1), a putative mannose-binding protein, targets misfolded glycoproteins for ERAD. We report that endogenous EDEM1 exists mainly as a soluble glycoprotein. By high-resolution immunolabeling and serial section analysis, we find that endogenous EDEM1 is sequestered in buds that form along cisternae of the rough ER at regions outside of the transitional ER. They give rise to ≈150-nm vesicles scattered throughout the cytoplasm that are lacking a recognizable COPII coat. About 87% of the immunogold labeling was over the vesicles and ≈ 11% over the ER lumen. Some of the EDEM1 vesicles also contain Derlin-2 and the misfolded Hong Kong variant of α-1-antitrypsin, a substrate for EDEM1 and ERAD. Our results demonstrate the existence of a vesicle budding transport pathway out of the rough ER that does not involve the canonical transitional ER exit sites and therefore represents a previously unrecognized passageway to remove potentially harmful misfolded luminal glycoproteins from the ER.

3.891 **WD40 protein Mda1 is purified with Dnm1 and forms a dividing ring for mitochondria before Dnm1 in Cyanidioschyzon merolae**
Nishida, K., Yagisawa, F., Kuroiwa, H., Yoshida, Y. and Kuroiwa, T.
PNAS, ***104(11)***, *4736-4741 (2007)*

Mitochondria are not produced *de novo* but are maintained by division. Mitochondrial division is a coordinated process of positioning and constriction of the division site and fission of double membranes, in which dynamin-related protein is believed to mediate outer membrane fission. Part of the mitochondrial

division machinery was purified from M phase-arrested *Cyanidioschyzon merolae* cells through biochemical fractionation. The dynamin-related protein Dnm1 was one of the two major proteins in the purified fraction and was accompanied by a newly identified protein CMR185C, named Mda1. Mda1 contained a predictable coiled-coil region and WD40 repeats, similarly to Mdv1 and Caf4 in yeasts. Immunofluorescence and immunoelectron microscopy showed that Mda1 localizes as a medial belt or ring on the mitochondrial outer surface throughout the division. The ring formation of Mda1 followed the plane of the ring of FtsZ, a protein that resides in the matrix. Dnm1 consistently colocalized with Mda1 only in the late stages of division. Mda1 protein was expressed through S to M phases and was phosphorylated specifically in M phase when Mda1 transformed from belt into foci and became colocalizing with Dnm1. Dephosphorylation of Mda1 *in vitro* increased its sedimentation coefficient, suggesting conformational changes of the macromolecule. Disassembly of the purified mitochondrial division machinery was performed by adding GTP to independently release Dnm1, suggesting that Mda1 forms a stable homo-oligomer by itself as a core structure of the mitochondrial division machinery.

3.892 The novel cargo Alcadein induces vesicle association of kinesin-1 motor components and activates axonal transport
Araki, Y. et al
EMBO J., ***26***, *1475-1486 (2007)*

Alcadeinα (Alcα) is an evolutionarily conserved type I membrane protein expressed in neurons. We show here that Alcα strongly associates with kinesin light chain ($K_D \approx 4–8 \times 10^{-9}$ M) through a novel tryptophan- and aspartic acid-containing sequence. Alcα can induce kinesin-1 association with vesicles and functions as a novel cargo in axonal anterograde transport. JNK-interacting protein 1 (JIP1), an adaptor protein for kinesin-1, perturbs the transport of Alcα, and the kinesin-1 motor complex dissociates from Alcα-containing vesicles in a JIP1 concentration-dependent manner. Alcα-containing vesicles were transported with a velocity different from that of amyloid β-protein precursor (APP)-containing vesicles, which are transported by the same kinesin-1 motor. Alcα- and APP-containing vesicles comprised mostly separate populations in axons *in vivo*. Interactions of Alcα with kinesin-1 blocked transport of APP-containing vesicles and increased β-amyloid generation. Inappropriate interactions of Alc- and APP-containing vesicles with kinesin-1 may promote aberrant APP metabolism in Alzheimer's disease.

3.893 Accumulation of Mutant Neuroserpin Precedes Development of Clinical Symptoms in Familial Encephalopathy with Neuroserpin Inclusion Bodies
Galliciotti, G. et al
Am. J. Pathol., ***170(4)***, *1305-1313 (2007)*

Intracellular protein deposition due to aggregation caused by conformational alteration is the hallmark of a number of neurodegenerative disorders, including Parkinson's disease, tauopathies, Huntington's disease, and familial encephalopathy with neuroserpin inclusion bodies. The latter is an autosomal dominant disorder caused by point mutations in neuroserpin resulting in its destabilization. Mutant neuroserpin polymerizes and forms intracellular aggregates that eventually lead to neurodegeneration. We generated genetically modified mice expressing the late-onset S49P-Syracuse or the early-onset S52R-Portland mutation of neuroserpin in central nervous system neurons. Mice exhibited morphological, biochemical, and clinical features resembling those found in the human disease. Analysis of brains revealed large intraneuronal inclusions composed exclusively of mutant neuroserpin, accumulating long before the development of clinical symptoms in a time-dependent manner. Clinical symptoms and amount of neuroserpin inclusions correlated with the predicted instability of the protein. The presence of inclusion bodies in subclinical mice indicates that in humans the prevalence of the disease could be higher than anticipated. In addition to shedding light on the pathophysiology of the human disorder, these mice provide an excellent model to study mechanisms of neurodegeneration or establish novel therapies for familial encephalopathy with neuroserpin inclusion bodies and other neurodegenerative diseases with intracellular protein deposition.

3.894 Cytosolic Activation of Cathepsins Mediates Parvovirus H-1-Induced Killing of Cisplatin and TRAIL-Resistant Glioma Cells
Di Piazza, M. et al
J. Virol., ***81(8)***, *4186-4198 (2007)*

Gliomas are often resistant to the induction of apoptotic cell death as a result of the development of survival mechanisms during astrocyte malignant transformation. In particular, the overexpression of Bcl-2-

family members interferes with apoptosis initiation by DNA-damaging agents (e.g., cisplatin) or soluble death ligands (e.g., TRAIL). Using low-passage-number cultures of glioma cells, we have shown that parvovirus H-1 is able to induce death in cells resistant to TRAIL, cisplatin, or both, even when Bcl-2 is overexpressed. Parvovirus H-1 triggers cell death through both the accumulation of lysosomal cathepsins B and L in the cytosol of infected cells and the reduction of the levels of cystatin B and C, two cathepsin inhibitors. The impairment of either of these effects protects glioma cells from the viral lytic effect. In normal human astrocytes, parvovirus H-1 fails to induce a killing mechanism. In vivo, parvovirus H-1 infection of rat glioma cells intracranially implanted into recipient animals triggers cathepsin B activation as well. This report identifies for the first time cellular effectors of the killing activity of parvovirus H-1 against malignant brain cells and opens up a therapeutic approach which circumvents their frequent resistance to other death inducers.

3.895 Knockdown of ACAT-1 reduces amyloidogenic processing of APP
Huttunen, H.J., Greco, C. and Kovacs, D.M.
FEBS Lett., ***581***, *1688-1692 (2007)*

Previous studies have shown that acyl-coenzyme A:cholesterol acyl transferase (ACAT), an enzyme that controls cellular equilibrium between free cholesterol and cholesteryl esters, modulates proteolytic processing of APP in cell-based and animal models of Alzheimer's disease. Here we report that ACAT-1 RNAi reduced cellular ACAT-1 protein by ~50% and cholesteryl ester levels by 22% while causing a slight increase in the free cholesterol content of ER membranes. This correlated with reduced proteolytic processing of APP and 40% decrease in Aβ secretion. These data show that even a modest decrease in ACAT activity can have robust suppressive effects on Aβ generation.

3.896 The Nicastrin-like Protein Nicalin Regulates Assembly and Stability of the Nicalin-Nodal Modulator (NOMO) Membrane Protein Complex
Haffner, C., Dettmer, U., Weiler, T. And Haass, C.
J. Biol. Chem., ***282(14)***, *10632-10638 (2007)*

The assembly of the γ-secretase complex, an Alzheimer disease-related protease required for β-amyloid generation, is tightly regulated and predominantly limited by the stoichiometrical availability of its components. We have identified a novel endoplasmic reticulum-located protein complex that is regulated in a similar fashion. It contains the recently identified Nodal signaling antagonists Nicalin (a distant homolog of the γ-secretase component Nicastrin) and NOMO (Nodal modulator). Using an RNA interference approach, we found that Nicalin and NOMO became unstable in the absence of the respective binding partner, suggesting that complex formation has a stabilizing effect. Overexpression of Nicalin resulted in an increase in NOMO, whereas endogenous Nicalin was reduced below the detection limit. Both effects were shown to occur at a post-transcriptional level. Thus, NOMO is most likely produced in excess amounts and either stabilized by Nicalin or rapidly degraded. In contrast, Nicalin levels are limited independently of NOMO. We, therefore, propose that Nicalin controls the assembly and stability of the Nicalin-NOMO complex.

3.897 Free Cholesterol Alters Lipid Raft Structure and Function Regulating Neutrophil Ca2+ Entry and Respiratory Burst: Correlations with Calcium Channel Raft Trafficking
Kannan, K.B., Barlos, D. and Hauser, C.J.
J. Immunol., ***178***, *5253-5261 (2007)*

Recent studies associate cholesterol excess and atherosclerosis with inflammation. The link between these processes is not understood, but cholesterol is an important component of lipid rafts. Rafts are thought to concentrate membrane signaling molecules and thus regulate cell signaling through G protein-coupled pathways. We used methyl β-cyclodextrin to deplete cholesterol from polymorphonuclear neutrophil (PMN) rafts and thus study the effects of raft disruption on G protein-coupled Ca^{2+} mobilization. Methyl β-cyclodextrin had no effect on Ca^{2+} store depletion by the G protein-coupled agonists platelet-activating factor or fMLP, but abolished agonist-stimulated Ca^{2+} entry. Free cholesterol at very low concentrations regulated Ca^{2+} entry into PMN via nonspecific Ca^{2+} channels in a biphasic fashion. The specificity of cholesterol regulation for Ca^{2+} entry was confirmed using thapsigargin studies. Responses to cholesterol appear physiologic because they regulate respiratory burst in a proportional biphasic fashion. Investigating further, we found that free cholesterol accumulated in PMN lipid raft fractions, promoting formation and polarization of membrane rafts. Finally, the transient receptor potential calcium channel protein TRPC1 redistributed to raft fractions in response to cholesterol. The uniformly biphasic relationships between

cholesterol availability, Ca^{2+} signaling and respiratory burst suggest that Ca^{2+} influx and PMN activation are regulated by the quantitative relationships between cholesterol and other environmental lipid raft components. The association between symptomatic cholesterol excess and inflammation may therefore in part reflect free cholesterol- dependent changes in lipid raft structure that regulate immune cell Ca^{2+} entry. Ca^{2+} entry-dependent responses in other cell types may also reflect cholesterol bioavailability and lipid incorporation into rafts.

3.898 Association of putative ammonium exporters Ato with detergent-resistant compartments of plasma membrane during yeast colony development: pH affects Ato1p localisation in patches
Ricicova, M., Kucerova, H., Vachova, L. and Palkova, Z.
*Biochim. Biophys. Acta, **1768**, 1170-1178 (2007)*

It was proposed that Ato1p, Ato2p and Ato3p have a role in ammonia production by *Saccharomyces cerevisiae* colonies (Palkova et al., Mol Biol Cell 13: 3901–3914, 2002). In this study, we show that all three Ato proteins localise to the plasma membrane and their appearance correlates with the beginning of ammonia release. The expression of *ATO* genes is controlled by ammonia. All three Ato–GFP proteins associate with detergent-resistant membranes; two of them, Ato1p–GFP and Ato3p–GFP, localise to patches visible under the fluorescence microscope. In contrast with Ato3p–GFP which forms stable patches, the formation of those of Ato1p–GFP is pH dependent. Ato1p–GFP patches form at pH above 6 and they disappear at pH 5 or lower. Both changes, Ato1p–GFP clustering and patches spreading are reversible. The Ato1p–GFP spreading at low pH is independent on endocytosis. These data suggest that besides the ammonia induction of Ato protein synthesis, pH may rapidly regulate Ato1p function.

3.899 Polybasic KKR Motif in the Cytoplasmic Tail of Nipah Virus Fusion Protein Modulates Membrane Fusion by Inside-Out Signaling
Aguilar, H et al
*J. Virol., **81(9)**, 4520-4532 (2007)*

The cytoplasmic tails of the envelope proteins from multiple viruses are known to contain determinants that affect their fusogenic capacities. Here we report that specific residues in the cytoplasmic tail of the Nipah virus fusion protein (NiV-F) modulate its fusogenic activity. Truncation of the cytoplasmic tail of NiV-F greatly inhibited cell-cell fusion. Deletion and alanine scan analysis identified a tribasic KKR motif in the membrane-adjacent region as important for modulating cell-cell fusion. The K1A mutation increased fusion 5.5-fold, while the K2A and R3A mutations decreased fusion 3- to 5-fold. These results were corroborated in a reverse-pseudotyped viral entry assay, where receptor-pseudotyped reporter virus was used to infect cells expressing wild-type or mutant NiV envelope glycoproteins. Differential monoclonal antibody binding data indicated that hyper- or hypofusogenic mutations in the KKR motif affected the ectodomain conformation of NiV-F, which in turn resulted in faster or slower six-helix bundle formation, respectively. However, we also present evidence that the hypofusogenic phenotypes of the K2A and R3A mutants were effected via distinct mechanisms. Interestingly, the K2A mutant was also markedly excluded from lipid rafts, where ~20% of wild-type F and the other mutants can be found. Finally, we found a strong negative correlation between the relative fusogenic capacities of these cytoplasmic-tail mutants and the avidities of NiV-F and NiV-G interactions ($P = 0.007$, $r^2 = 0.82$). In toto, our data suggest that inside-out signaling by specific residues in the cytoplasmic tail of NiV-F can modulate its fusogenicity by multiple distinct mechanisms.

3.900 Participation of Rab5, an Early Endosome Protein, in Hepatitis C Virus RNA Replication Machinery
Stone, M., Jia, S., Do Heo, W., Meyer, T. and Konan, K.V.
*J. Virol., **81(9)**, 4551-4563 (2007)*

Like most positive-strand RNA viruses, hepatitis C virus (HCV) is believed to replicate its genome on the surface of rearranged membranes. We have shown previously that HCV NS4AB, but not the product NS4B, inhibits endoplasmic reticulum (ER)-to-Golgi protein traffic (K. V. Konan, T. H. Giddings, Jr., M. Ikeda, K. Li, S. M. Lemon, and K. Kirkegaard, J. Virol. 77:7843-7855). However, both NS4AB and NS4B can induce "membranous web" formation, first reported by Egger et al. (D. B Egger, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz, J. Virol. 76:5974-5984), which is also observed in HCV-infected cells (Y. Rouille, F. Helle, D. Delgrange, P. Roingeard, C. Voisset, E. Blanchard, S. Belouzard, J. McKeating, A. H. Patel, G. Maertens, T. Wakita, C. Wychowski, and J. Dubuisson, J. Virol. 80:2832-2841) and cells that bear a subgenomic NS5A-green fluorescent protein (GFP) replicon (D. Moradpour, M.

J. Evans, R. Gosert, Z. Yuan, H. E. Blum, S. P. Goff, B. D. Lindenbach, and C. M. Rice, J. Virol. 78:7400-7409). To determine the intracellular origin of the web, we examined NS4B colocalization with endogenous cellular markers in the context of the full-length or subgenomic replicon. We found that, in addition to ER markers, early endosome (EE) proteins, including Rab5, were associated with web-inducing protein NS4B. Furthermore, an immunoisolated fraction containing NS4B was found to contain both ER and EE proteins. Using fluorescence microscopy, we showed that wild-type and constitutively active Rab5 proteins were associated with NS4B. Interestingly, expression of dominant-negative Rab5 resulted in significant loss of GFP fluorescence in NS5A-GFP replicon cells. We also found that a small reduction in Rab5 protein expression decreased HCV RNA synthesis significantly. Furthermore, transfection of labeled Rab5 small interfering RNAs into NS5A-GFP replicon cells resulted in a significant decrease in GFP fluorescence. Finally, Rab5 protein was found to coimmunoprecipitate with HCV NS4B. These studies suggest that EE proteins, including Rab5, may play a role in HCV genome replication or web formation.

3.901 ***Measles virus*** **nucleocapsid transport to the plasma membrane requires stable expression and surface accumulation of the viral matrix protein**
Runkler, N., Pohl, C., Schneider-Schaulies, S., Klenk, H-D. and Maisner, A.
Cell. Microbiol., ***9(5)****, 1203-1214 (2007)*

In measles virus (MV)-infected cells the matrix (M) protein plays a key role in virus assembly and budding processes at the plasma membrane because it mediates the contact between the viral surface glycoproteins and the nucleocapsids. By exchanging valine 101, a highly conserved residue among all paramyxoviral M proteins, we generated a recombinant MV (rMV) from cloned cDNA encoding for a M protein with an increased intracellular turnover. The mutant rMV was barely released from the infected cells. This assembly defect was not due to a defective M binding to other matrix- or nucleoproteins, but could rather be assigned to a reduced ability to associate with cellular membranes, and more importantly, to a defective accumulation at the plasma membrane which was accompanied by the deficient transport of nucleocapsids to the cell surface. Thus, we show for the first time that M stability and accumulation at intracellular membranes is a prerequisite for M and nucleocapsid co-transport to the plasma membrane and for subsequent virus assembly and budding processes.

3.902 **Establishment of subcellular fractionation techniques to monitor the intracellular fate of polymer therapeutics II. Identification of endosomal and lysosomal compartments in HepG2 cells combining single-step subcellular fractionation with fluorescent imaging**
Manunta, M., Izzo, L., Duncan, R. and Jones, A.T.
J. Drug. Target., ***15(1)****, 37-50 (2007)*

As they are often designed for lysosomotropic, endosomotropic and/or transcellular delivery, an understanding of intracellular trafficking pathways is essential to enable optimised design of novel polymer therapeutics. Here, we describe a single-step density gradient subcellular fractionation method combined with fluorescent detection analysis that provides a new tool for characterisation of endocytic traffic of polymer therapeutics. Hepatoma (HepG2) cells were used as a model and cell breakage was optimised using a cell cracker to ensure assay of the whole cell population. After removal of unbroken cells and nuclei, the cell lysate as a post-nuclear supernatant (PNS) was layered onto an iodixanol (OptiPrep) density gradient optimised to 5-20%. Early endosomes, late endosomes and lysosomes were identified from gradient fractions by immunoblotting for marker proteins early endosome antigen 1 (EEA 1) and lysosomal associated membrane protein 1 (LAMP 1) using horseradish peroxidase or fluorescently-labelled secondary antibodies. Lysosomes were also detected using N-acetyl-beta-glucosamindase (Hex A) activity. In addition, cells were incubated with Texas-red labelled transferrin (TxR-Tf) for 5 min to specifically label early endosomes and this was directly detected from SDS-PAGE gels. Internalised macromolecules and colloidal particles can potentially alter vesicle buoyant density. To see if typical macromolecules of interest would alter vesicle density or perturb vesicle traffic, HepG2 cells were incubated with dextran or a polyethyleneglycol (PEG)-polyester dendron G4 (1 mg/ml for 24 h). The PEG-polyester dendron G4 caused a slight redistribution of endocytic structures to lower density fractions but immunofluorescence microscopy showed no obvious dendron effects. In conclusion, the combined subcellular fractionation with fluorescent imaging approach described here can be used as a tool for both fundamental cell biology research and/or the quantitative localisation of polymer therapeutics in the endocytic pathway.

3.903 Differential stimulation-induced receptor localization in lipid rafts for interleukin-6 family cytokines signaling through the gp130/leukemia inhibitory factor receptor complex
Port, M.D., Gibson, R.-M. and Nathanson, N.M.
J. Neurochem., ***101***, *782-793 (2007)*

Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are cytokines which signal through receptor complexes that include the receptor subunits glycoprotein 130 (gp130) and the LIF receptor (LIFR), but CNTF also requires the non-signal transducing CNTF receptor (CNTFR) for binding. We show here that in IMR-32 neuronal cells endogenously expressing the receptor subunits for LIF and CNTF, CNTFR, but not gp130 or LIFR, is found in detergent-resistant lipid rafts. In addition, stimulation of these cells with CNTF resulted in a rapid translocation of a portion of gp130 and LIFR into detergent-resistant lipid rafts while an equivalent stimulation with LIF did not. Disruption of lipid rafts by cholesterol depletion of cell membranes blocked the CNTF-induced translocation of LIFR and gp130. Interestingly, while cholesterol-depletion did not inhibit signal transducer and activator of transcription 3 phosphorylation by either CNTF or LIF stimulation, it strongly inhibited both CNTF- and LIF-mediated phosphorylation of extracellular signal-regulated kinases 1 and 2 and Akt. LIF and CNTF generally appear to have redundant effects in cells responsive to both cytokines. Intriguingly, the data presented here suggest a possible mechanism whereby CNTF or other cytokines that signal through CNTFR could generate signals distinct from those elicited by cytokines such as LIF which utilize a LIFR/gp130 heterodimer, via association with or exclusion from lipid rafts.

3.904 Molecular characterization of GDD1/TMEM16E, the gene product responsible for autosomal dominant gnathodiaphyseal dysplasia
Mizuta, K. et al
Biochem. Biophys. Acta, ***357***, *126-132 (2007)*

The human *GDD1/TMEM16E* gene has been found to be mutated in *gnathodiaphyseal dysplasia*, an unusual skeletal syndrome with autosomal dominant inheritance. The molecular and biochemical function(s) of GDD1 protein has not yet been elucidated. In this study, we examined the murine *GDD1* gene expression pattern during embryonic development, and characterized the cellular and tissue localizations of its gene product using a GDD1-specific antibody. In the developing embryos, *GDD1* mRNA expression was principally associated with differentiating and developing somites, with a highly complex spatiotemporal pattern that involved the myotomal and sclerotomal lineages of somites. Biochemical studies indicated that GDD1 protein is an integral membrane glycoprotein that resides predominantly in intracellular vesicles. Immunohistochemical analysis showed a high level of murine GDD1 protein expression in cardiac and skeletal muscle tissues, and in growth-plate chondrocytes and osteoblasts in bone. These observations suggest diverse cellular role(s) of GDD1 in the development of musculoskeletal system.

3.905 UK114, a YjgF/Yer057p/UK114 family protein highly conserved from bacteria to mammals, is localized in rat liver peroxisomes
Antonenkov, V., Ohlmeier, S., Sormunen, R.T. and Hiltunen, J.K.
Biochem. Biophys. Acta, ***357***, *252-257 (2007)*

Mammalian UK114 belongs to a highly conserved family of proteins with unknown functions. Although it is believed that UK114 is a cytosolic or mitochondrial protein there is no detailed study of its intracellular localization. Using analytical subcellular fractionation, electron microscopic colloidal gold technique, and two-dimensional gel electrophoresis of peroxisomal matrix proteins combined with mass spectrometric analysis we show here that a large portion of UK114 is present in rat liver peroxisomes. The peroxisomal UK114 is a soluble matrix protein and it is not inducible by the peroxisomal proliferator clofibrate. The data predict involvement of UK114 in peroxisomal metabolism.

3.906 Fis1, DLP1, and Pex11p coordinately regulate peroxisome morphogenesis
Kobayashi, S., Tanaka, A. and Fujika, Y.
Exp. Cell Res., ***313***, *1675-1686 (2007)*

Dynamin-like protein 1 (DLP1) and Pex11pβ function in morphogenesis of peroxisomes. In the present work, we investigated whether Fis1 is involved in fission of peroxisomes. Endogenous Fis1 was morphologically detected in peroxisomes as well as mitochondria in wild-type CHO-K1 and *DLP1*-defective ZP121 cells. Subcellular fractionation studies also revealed the presence of Fis1 in peroxisomes.

Peroxisomal Fis1 showed the same topology, i.e., C-tail anchored membrane protein, as the mitochondrial one. Furthermore, ectopic expression of *FIS1* induced peroxisome proliferation in CHO-K1 cells, while the interference of *FIS1* RNA resulted in tubulation of peroxisomes, hence reducing the number of peroxisomes. Fis1 interacted with Pex11pβ, by direct binding apparently involving the C-terminal region of Pex11pβ in the interaction. Pex11pβ also interacted with each other, whereas the binding of Pex11pβ to DLP1 was not detectable. Moreover, ternary complexes comprising Fis1, Pex11pβ, and DLP1 were detected by chemical cross-linking. We also showed that the highly conserved N-terminal domain of Pex11pβ was required for the homo-oligomerization of Pex11pβ and indispensable for the peroxisome-proliferating activity. Taken together, these findings indicate that Fis1 plays important roles in peroxisome division and maintenance of peroxisome morphology in mammalian cells, possibly in a concerted manner with Pex11pβ and DLP1.

3.907 Opposing Actions of Endocannabinoids on Cholangiocarcinoma Growth: RECRUITMENT OF Fas AND Fas LIGAND TO LIPID RAFTS
DeMorrow, S. et al
J. Biol. Chem., ***282(17)***, *13098-13113 (2007)*

Cholangiocarcinomas are devastating cancers of biliary origin with limited treatment options. Modulation of the endocannabinoid system is being targeted to develop possible therapeutic strategies for a number of cancers; therefore, we evaluated the effects of the two major endocannabinoids, anandamide and 2-arachidonylglycerol, on numerous cholangiocarcinoma cell lines. Although anandamide was antiproliferative and proapoptotic, 2-arachidonylglycerol stimulated cholangiocarcinoma cell growth. Specific inhibitors for each of the cannabinoid receptors did not prevent either of these effects nor did pretreatment with pertussis toxin, a $G_{i/o}$ protein inhibitor, suggesting that anandamide and 2-arachidonylglycerol did not exert their diametric effects through any known cannabinoid receptor or through any other $G_{i/o}$ protein-coupled receptor. Using the lipid raft disruptors methyl-β-cyclodextrin and filipin, we demonstrated that anandamide, but not 2-arachidonylglycerol, requires lipid raft-mediated events to inhibit cellular proliferation. Closer inspection of the lipid raft structures within the cell membrane revealed that although anandamide treatment had no observable effect 2-arachidonylglycerol treatment effectively dissipated the lipid raft structures and caused the lipid raft-associated proteins lyn and flotillin-1 to disperse into the surrounding membrane. In addition, anandamide, but not 2-arachidonylglycerol, induced an accumulation of ceramide, which was required for anandamide-induced suppression of cell growth. Finally we demonstrated that anandamide and ceramide treatment of cholangiocarcinoma cells recruited Fas and Fas ligand into the lipid rafts, subsequently activating death receptor pathways. These findings suggest that modulation of the endocannabinoid system may be a target for the development of possible therapeutic strategies for the treatment of this devastating cancer.

3.908 RhoB plays an essential role in CXCR2 sorting decisions
Neel, N.F., Lapierre, L.A., Goldenring, J.R. and Richmond, A.
J. Cell Sci., ***120***, *1559-1571 (2007)*

The CXCR2 chemokine receptor is a G-protein-coupled receptor that undergoes clathrin-mediated endocytosis upon ligand binding. The trafficking of CXCR2 is crucial for cells to maintain a proper chemotactic response. The mechanisms that regulate the recycling/degradation sorting decision are unknown. In this study, we used dominant-negative (T19N) and GTPase-deficient activated (Q63L) RhoB mutants, as well as RhoB small interfering RNA (siRNA) to investigate the role of RhoB in CXCR2 trafficking. Expression of either of the RhoB mutants or transfection of RhoB siRNA impaired CXCR2-mediated chemotaxis. Expression of RhoB T19N and transfection of RhoB siRNA impaired sorting of CXCR2 to the lysosome after 3 hours of CXCL8 stimulation and impaired CXCL8-induced CXCR2 degradation. In cells expressing the RhoB Q63L mutant, CXCR2 recycling through the Rab11a recycling compartment was impaired after 30 minutes of CXCL8 stimulation as was CXCL8-induced CXCR2 degradation. For cells expressing activated RhoB, CXCR2 colocalized with Rab4, a marker for the rapid recycling pathway, and with the mannose-6-phosphate receptor, which traffics between the trans-Golgi network and endosomes. These data suggest that CXCR2 recycles through alternative pathways. We conclude that oscillation of RhoB GTPase activity is essential for appropriate sorting decisions, and for directing CXCR2 degradation and recycling – events that are required for optimal chemotaxis.

3.909 Tyrosine phosphorylation and lipid raft association of pseudorabies virus glycoprotein E during antibody-mediated capping
Desplanques, A.S., Nauwynck, H.J., Tilleman, K., Deforce, D. and Favoreel, H.W.

Virology, ***362(1)***, *60-66 (2007)*

In specific cell types infected with the alphaherpesviruses herpes simplex virus and pseudorabies virus (PRV), addition of virus-specific antibodies results in redistribution of cell-surface-anchored viral proteins. This redistribution is triggered by the viral protein gE and consists of the directional movement of the antibody–antigen complexes to one pole of the cell. This viral capping process has been associated with increased antibody-resistant virus spread and strongly resembles immunoreceptor capping, a process that is crucial in activation of different immune cells (e.g. capping of Fcγ-receptors, B and T cell receptors). Here, we report that the PRV gE-mediated viral capping process results in increased Src kinase-mediated tyrosine phosphorylation of the cytoplasmic domain of gE and that a fraction of gE associates with lipid rafts, all very reminiscent of immunoreceptor capping. These results provide evidence that gE-mediated capping is a viral mimicry of immunoreceptor capping.

3.910 APOBEC3G Multimers Are Recruited to the Plasma Membrane for Packaging into Human Immunodeficiency Virus Type 1 Virus-Like Particles in an RNA-Dependent Process Requiring the NC Basic Linker
Burnett, A. and Spearman, P.
J. Virol., ***81(10)***, *5000-5013 (2007)*

APOBEC3G is an endogenous host restriction factor that inhibits human immunodeficiency virus (HIV) replication. The antiviral activity of APOBEC3G is dependent upon its incorporation into the virus particle. The mechanisms governing incorporation of APOBEC3G into virus particles are not completely understood. In particular, some investigators have reported that APOBEC3G interacts directly with the nucleocapsid (NC) subunit of Gag, while others have found that an RNA intermediate is required for Gag-APOBEC3G interactions. In this study, we confirmed the RNA dependence of APOBEC3G packaging and performed detailed mapping of the determinants within NC that are required for virion incorporation. Surprisingly, APOBEC3G packaging did not correlate well with the presence of the N-terminal "I," or interaction, domain within NC. Specifically, Gag constructs containing only the N-terminal region of NC packaged minimal amounts of APOBEC3G, while significant levels of APOBEC3G packaging were achieved with Gag constructs containing the basic linker region of NC. Furthermore, membrane-binding experiments revealed that the basic linker region was essential for the membrane association of APOBEC3G in a Gag-APOBEC3G complex. Fluorescence resonance energy transfer was detected between labeled APOBEC3G in cells and in particles, indicating that APOBEC3G is packaged as a multimer that is bound to packaged RNA. Regions of APOBEC3G-Gag colocalization at the plasma membrane were detected that were distinct from the punctate cytoplasmic bodies where APOBEC3G accumulates within the cell. Together, our results indicate that APOBEC3G multimerizes in an RNA-dependent fashion and that RNA-APOBEC3G multimers are recruited to the plasma membrane and subsequently into virion particles by Gag.

3.911 The Tyrosine Kinase Fyn Determines the Localization of TrkB Receptors in Lipid Rafts
Pereira, D.R. and Chao, M.V.
J. Neurosci., ***27(18)***, *4859-4869 (2007)*

Localization of Trk neurotrophin receptors is an important factor in directing cellular communication in developing and mature neurons. One potential site of action is in lipid raft membrane microdomains. Although Trk receptors have been localized to lipid rafts, little is known about how these neurotrophin receptors are directed there or how localization to these membrane microdomains regulates Trk signaling. Here, we report that the TrkB brain-derived neurotrophic factor (BDNF) receptor specifically localized to intracellular lipid rafts in cortical and hippocampal membranes in response to BDNF and that this process was critically dependent on the tyrosine kinase Fyn. BDNF-induced TrkB accumulation at lipid rafts was prevented by blocking the internalization of TrkB. BDNF stimulation also resulted in the association between endogenous TrkB and Fyn. Moreover, in neurons derived from Fyn knock-out mice, the translocation of TrkB to lipid rafts in response to BDNF was compromised, whereas the corticohippocampal region of Fyn mutants displayed lower amounts of TrkB in lipid rafts *in vivo*. In support of a role for lipid rafts in neurotrophin signaling, inhibiting TrkB translocation to lipid rafts, either by using Fyn knock-out neurons or lipid raft-disturbing agents, prevented the full activation of TrkB and of downstream phospholipase C-γ. These results indicate that the lipid raft localization of TrkB receptors is regulated by Fyn and represents an important factor in determining the outcome of BDNF signaling in neurons.

3.912 Coupling of the de Novo Fatty Acid Biosynthesis and Lipoylation Pathways in Mammalian Mitochondria

Witkowski, A., Joshi, A. and Smith, S.
J. Biol. Chem., ***282(19)***, *14178-14185 (2007)*

The objective of this study was to identify the products and possible role of a putative pathway for *de novo* fatty acid synthesis in mammalian mitochondria. Bovine heart mitochondrial matrix preparations were prepared free from contamination by proteins from other subcellular components and, using a combination of radioisotopic labeling and mass spectrometry, were shown to contain all of the enzymes required for the extension of a 2-carbon precursor by malonyl moieties to saturated acyl-ACP thioesters containing up to 14 carbon atoms. A major product was octanoyl-ACP and, in the presence of the apo-H-protein of the glycine cleavage complex, the newly synthesized octanoyl moieties were translocated to the lipoylation site on the acceptor protein. These studies demonstrate that one of the functions of the *de novo* fatty acid biosynthetic pathway in mammalian mitochondria is to provide the octanoyl precursor required for the essential protein lipoylation pathway.

3.913 Separation of cell-cell adhesion complexes by differential centrifugation

Vogelmann, R. and Nelson, W.J.
Methods in Mol. Biol., ***370***, *11-22 (2007)*

The number of proteins found associated with cell-cell adhesion substructures is growing rapidly. Based on potential protein-protein interactions, complex protein networks at cell-cell contacts can be modeled. Traditional studies to examine protein-protein interactions include co-immunoprecipitation or pull-down experiments of tagged proteins. These studies provide valuable information that proteins can associate directly or indirectly through other proteins in a complex. However, they do not clarify if a given protein is part of other protein complexes or inform about the specificity of those interactions in the context of adhesion substructures. Thus, it is not clear if models compiled from these types of studies reflect the combination of protein interactions in the adhesion complex in vivo for a specific cell type. Therefore, we present here a method to separate cell-cell contact membrane substructures with their associated protein complexes based on their buoyant behavior in iodixanol density gradients. Analysis of 16 proteins of the apical junctional complex (AJC) in epithelial Madin-Darby canine kidney cells revealed a more simple organization of the AJC adhesion complex than that predicted from the combination of all possible protein-protein interactions defined from co-immunoprecipitation and pull-down experiments.

3.914 HSV Viral Envelope Proteins Partition With Lipid Rafts in Infected Retinal Ganglion Cell Axons

Cortez, D.A., Sucher, A. and LaVail, J.H.
Invest. Ophthalmol. Vis. Sci., ***48***, *E-abstract 3166 (2007)*

Purpose: Herpes simplex virus type 1 (HSV) is responsible for recurrent scarring of the corneal epithelium in ocular herpetic keratitis, a common cause of blindness. HSV envelope glycoproteins are essential for cell-cell spread of infection from trigeminal axons to corneal cells. What has been lacking is details of how newly made envelope proteins are transported within axons. By analogy to the transport of synaptic membrane precursors, lipid raft membranes may also support the transport of viral glycoproteins. We have tested the hypothesis that HSV glycoproteins are transported in association with lipid raft membranes in infected retinal axons in vivo.
Methods: Murine retinal ganglion cells were infected with a wild-type (wt) virus, and after 24 hrs the mice were given Valacyclovir to pulse infect the neurons. The optic pathways were dissected 5 days postinfection, and the tissues were processed for Western blotting using antibodies to gB, gC and gD. Additional animals were infected and treated as above, but the optic pathways were dissected and prepared in an **Optiprep** flotation assay to separate detergent-resistant membranes (DRM) and detergent-soluble membranes (DSM). We used GM1 and caveolin as control proteins for DRM and transferrin receptor as a control for DSM.
Results: By 5 days postinfection all three glycoproteins were transported to the OT. However, the transport of gD appeared to be more efficient than that of gC or gB. In preliminary studies all of the tested glycoproteins, (gB, gC, and gD) as well as GM1 and caveolin were present in the DRM fractions. We also found gB, gC and gD in the DSM fraction. Transferrin receptor was found principally in the DSM fraction.
Conclusions: By five days after infection the three glycoproteins had been transported to the most distal portion of the retinal axons. The three envelope glycoproteins associated with the lipid raft membrane fraction. Further experiments to define host membrane components that associate with transported viral glycoproteins will be essential to understanding the intracellular localization and mechanisms of transport.

3.915 Myelin protein zero/P0 phosphorylation and function require an adaptor protein linking it to RACK1 and PKCα
Gaboreanu, A-M. et al
J. Cell Biol., ***177(4)***, *707-716 (2007)*

Point mutations in the cytoplasmic domain of myelin protein zero (P0; the major myelin protein in the peripheral nervous system) that alter a protein kinase Cα (PKCα) substrate motif (198HRSTK201) or alter serines 199 and/or 204 eliminate P0-mediated adhesion. Mutation in the PKCα substrate motif (R198S) also causes a form of inherited peripheral neuropathy (Charcot Marie Tooth disease [CMT] 1B), indicating that PKCα-mediated phosphorylation of P0 is important for myelination. We have now identified a 65-kD adaptor protein that links P0 with the receptor for activated C kinase 1 (RACK1). The interaction of p65 with P0 maps to residues 179–197 within the cytoplasmic tail of P0. Mutations or deletions that abolish p65 binding reduce P0 phosphorylation and adhesion, which can be rescued by the substitution of serines 199 and 204 with glutamic acid. A mutation in the p65-binding sequence G184R occurs in two families with CMT, and mutation of this residue results in the loss of both p65 binding and adhesion function.

3.916 Characterization of Mammalian Par 6 as a Dual-Location Protein
Cline, E.G. and Nelson, W.J.
Mol. Cell. Biol., ***27(12)***, *4431-4443 (2007)*

Par 6 acts as a scaffold protein to facilitate atypical protein kinase C-mediated phosphorylation of cytoplasmic protein complexes, leading to epithelial and neuronal cell polarization. In addition to its location in the cytoplasm, Par 6 is localized to the nucleus. However, its organization and potential functions in the nucleus have not been examined. Using an affinity-purified Par 6 antibody and a chimera of Par 6 and green fluorescent protein, we show that Par 6 localizes precisely to nuclear speckles, but not to other nuclear structures, and displays characteristics of speckle proteins. We show that Par 6 colocalizes in the nucleus with Tax, a transcriptional activator of the human T-cell leukemia virus type 1 long terminal repeat, but multiple lines of evidence show that Par 6 is not directly involved in known functions of speckle proteins, including general transcription, splicing, or mRNA transport. Significantly, however, the structure of nuclear speckles is lost when Par 6 levels are reduced by Par 6-specific small interfering RNA. Therefore, we hypothesize that Par 6 in the nucleus acts as a scaffolding protein in nuclear speckle complexes, similar to its role in the cytoplasm.

3.917 The Host Protein Staufen1 Participates in Human Immunodeficiency Virus Type 1 Assembly in Live Cells by Influencing pr55Gag Multimerization
Chatel-Chaix, L., Abrahamyan, L., Frechima, C., Mouland, A.J. and DesGroseillers, L.
J. Virol., ***81(12)***, *6216-6230 (2007)*

Human immunodeficiency virus type 1 (HIV-1) requires the sequential activities of virus-encoded proteins during replication. The activities of several host cell proteins and machineries are also critical to the completion of virus assembly and the release of infectious virus particles from cells. One of these proteins, the double-stranded RNA-binding protein Staufen1 (Stau1), selectively associates with the HIV-1 genomic RNA and the viral precursor Gag protein, $pr55^{Gag}$. In this report, we tested whether Stau1 modulates $pr55^{Gag}$ assembly using a new and specific $pr55^{Gag}$ oligomerization assay based on bioluminescence resonance energy transfer (BRET) in both live cells and extracts after cell fractionation. Our results show that both the overexpression and knockdown of Stau1 increase the $pr55^{Gag}$-$pr55^{Gag}$ BRET levels, suggesting a role for Stau1 in regulating $pr55^{Gag}$ oligomerization during assembly. This effect of Stau1 on $pr55^{Gag}$ oligomerization was observed only in membranes, a cellular compartment in which $pr55^{Gag}$ assembly primarily occurs. Consistently, expression of Stau1 harboring a vSrc myristylation signal led to a 6.5-fold enrichment of Stau1 in membranes and a corresponding enhancement in the Stau1-mediated effect on $pr55^{Gag}$-$pr55^{Gag}$ BRET, demonstrating that Stau1 acts on assembly when targeted to membranes. A role for Stau1 in the formation of particles is further supported by the detection of membrane-associated detergent-resistant $pr55^{Gag}$ complexes and the increase of virus-like particle release when Stau1 expression levels are modulated. Our results indicate that Stau1 influences HIV-1 assembly by modulating $pr55^{Gag}$-$pr55^{Gag}$ interactions, as shown in a live cell interaction assay. This likely occurs when Stau1 interacts with membrane-associated assembly intermediates.

3.918 Binding Dynamics of Hepatitis C Virus' NS5A Amphipathic Peptide to Cell and Model Membranes
Cho, N-J., Cheong, K.H., Lee, CH., Frank, C.F. and Glenn, J.S.

J. Virol., ***81(12)***, *6682-6689 (2007)*

Membrane association of the hepatitis C virus NS5A protein is required for viral replication. This association is dependent on an N-terminal amphipathic helix (AH) within NS5A and is restricted to a subset of host cell intracellular membranes. The mechanism underlying this specificity is not known, but it may suggest a novel strategy for developing specific antiviral therapy. Here we have probed the mechanistic details of NS5A AH-mediated binding to both cell-derived and model membranes by use of biochemical membrane flotation and quartz crystal microbalance (QCM) with dissipation. With both assays, we observed AH-mediated binding to model lipid bilayers. When cell-derived membranes were coated on the quartz nanosensor, however, significantly more binding was detected, and the QCM-derived kinetic measurements suggested the existence of an interacting receptor in the target membranes. Biochemical flotation assays performed with trypsin-treated cell-derived membranes exhibited reduced AH-mediated membrane binding, while membrane binding of control cytochrome b5 remained unaffected. Similarly, trypsin treatment of the nanosensor coated with cellular membranes abolished AH peptide binding to the cellular membranes but did not affect the binding of a control lipid-binding peptide. These results therefore suggest that a protein plays a critical role in mediating and stabilizing the binding of NS5A's AH to its target membrane. These results also demonstrate the successful development of a new nanosensor technology ideal both for studying the interaction between a protein and its target membrane and for developing inhibitors of that interaction.

3.919 Membrane microdomain formation is crucial in epiboly during gastrulation of medaka
Adachi, T., Sato, C. and Kitajima, K.
Biochem. Biophys. Res. Comm., ***358(3)***, *848-853 (2007)*

Membrane microdomain (microdomain) was isolated from early gastrula embryos. The isolated microdomain was characterized by enrichment of cholesterol and sphingomyelin, and by the presence of huge glycoproteins containing Lewis X structure. Importance of the microdomain in the progress of epiboly was assessed using methyl β-cyclodextrin (MBCD) and C2-ceramide that disrupt microdomains through different mechanisms. Both reagents efficiently disrupted the microdomain structure and concomitantly impaired epiboly. Interestingly, when embryos pretreated with MBCD, a cholesterol-binding molecule, were exogenously supplemented with cholesterol, the embryos underwent not only reconstitution of the microdomain, but also complete restoration to the normal epiboly. Thus, normal or impaired development is reversibly controlled by the cholesterol-dependent formation or disruption of microdomains. The most typical phenotype of the microdomain-disrupted embryos is detachment of cells from the blastoderm, suggesting that a major contribution of microdomains to epiboly is cell adhesion of blastodermal cells.

3.920 Quantification and regulation of the subcellular distribution of bile acid coenzyme A:amino acid N-acyltransferase activity in rat liver
Styles, N.A., Falany, J.L., Barnes, S. and Falany, C.N.
J. Lipid Res., ***48***, *1305-1315 (2007)*

Bile acid coenzyme A:amino acid N-acyltransferase (BAT) is responsible for the amidation of bile acids with the amino acids glycine and taurine. To quantify total BAT activity in liver subcellular organelles, livers from young adult male and female Sprague-Dawley rats were fractionated into multiple subcellular compartments. In male and female rats, 65–75% of total liver BAT activity was found in the cytosol, 15–17% was found in the peroxisomes, and 5–10% was found in the heavy mitochondrial fraction. After clofibrate treatment, male rats displayed an increase in peroxisomal BAT specific activity and a decrease in cytosolic BAT specific activity, whereas females showed an opposite response. However, there was no overall change in BAT specific activity in whole liver homogenate. Treatment with rosiglitazone or cholestyramine had no effect on BAT activity in any subcellular compartment. These experiments indicate that the majority of BAT activity in the rat liver resides in the cytosol. Approximately 15% of BAT activity is present in the peroxisomal matrix. These data support the novel finding that clofibrate treatment does not directly regulate BAT activity but does alter the subcellular localization of BAT.

3.921 Contact-dependent inhibition of EGFR signaling by Nf2/Merlin
Curto, M., Cole, B.K., Lallemand, D., Liu, C-H. and McClatchey, A.I.
J. Cell Biol., ***177(5)***, *893-903 (2007)*

The neurofibromatosis type 2 (NF2) tumor suppressor, Merlin, is a membrane/cytoskeleton-associated protein that mediates contact-dependent inhibition of proliferation. Here we show that upon cell-cell contact Merlin coordinates the processes of adherens junction stabilization and negative regulation of epidermal growth factor receptor (EGFR) signaling by restraining the EGFR into a membrane compartment from which it can neither signal nor be internalized. In confluent *Nf2*$^{-/-}$ cells, EGFR activation persists, driving continued proliferation that is halted by specific EGFR inhibitors. These studies define a new mechanism of tumor suppression, provide mechanistic insight into the poorly understood phenomenon of contact-dependent inhibition of proliferation, and suggest a therapeutic strategy for *NF2*-mutant tumors.

3.922 Three-dimensional architecture of murine rod outer segments determined by cryoelectron tomography
Nickel, S., Park, P.S-H., Baumeister, W. and Polczewski, K.
J. Cell Biol., ***177(5)***, *917-925 (2007)*

The rod outer segment (ROS) of photoreceptor cells houses all components necessary for phototransduction, a set of biochemical reactions that amplify and propagate a light signal. Theoretical approaches to quantify this process require precise information about the physical boundaries of the ROS. Dimensions of internal structures within the ROS of mammalian species have yet to be determined with the precision required for quantitative considerations. Cryoelectron tomography was utilized to obtain reliable three-dimensional morphological information about this important structure from murine retina. Vitrification of samples permitted imaging of the ROS in a minimally perturbed manner and the preservation of substructures. Tomograms revealed the characteristic highly organized arrangement of disc membranes stacked on top of one another with a surrounding plasma membrane. Distances among the various membrane components of the ROS were measured to define the space available for phototransduction to occur. Reconstruction of segments of the ROS from single-axis tilt series images provided a glimpse into the three-dimensional architecture of this highly differentiated neuron. The reconstructions revealed spacers that likely maintain the proper distance between adjacent discs and between discs and the plasma membrane. Spacers were found distributed throughout the discs, including regions that are distant from the rim region of discs.

3.923 Role of caveolae in the pathogenesis of cholesterol-induced gallbladder muscle hypomotility
Xiao, Z., Schmitz, F., Pricolo, V.E., Biancani, P. and Behar, J.
Am. J. Physiol. Gastrointest. Liver Physiol., ***292***, *G1641-G1649 (2007)*

Muscle cells from human gallbladders (GB) with cholesterol stones (ChS) exhibit a defective contraction, excess cholesterol (Ch) in the plasma membrane, and lower binding of CCK-1 receptors. These abnormalities improved after muscle cells were incubated with Ch-free liposomes that remove the excess Ch from the plasma membrane. The present studies were designed to investigate the role of caveolin-3 proteins (Cav-3) in the pathogenesis of these abnormalities. Muscle cells from GB with ChS exhibit higher Ch levels in the plasma membrane that were mostly localized in caveolae and associated with parallel increases in the expression of Cav-3 in the caveolae compared with that in GB with pigment stones (PS). The overall number of CCK-1 receptors in the plasma membrane was not different between muscle cells from GB with ChS and PS, but they were increased in the caveolae in muscle cells from GB with ChS. Treatment of muscle cells from GB with ChS with a $G\alpha_{i3}$ protein fragment increased the total binding of CCK-1 receptors (from 8.3 to 11.2%) and muscle contraction induced by CCK-8 (from 11.2 to 17.3% shortening). However, $G\alpha_{q/11}$ protein fragment had no such effect. Moreover, neither fragment had any effect on muscle cells from GB with PS. We conclude that the defective contraction of muscle cells with excessive Ch levels in the plasma membrane is due to an increased expression of Cav-3 that results in the sequestration of CCK-1 receptors in the caveolae, probably by inhibiting the functions of $G\alpha_{i3}$ proteins.

3.924 Subcellular Localization and Physiological Significance of Intracellular Mannan-binding Protein
Nonaka, M. et al
J. Biol. Chem., ***282(24)***, *17908-17920 (2007)*

Mannan-binding protein (MBP) is a C-type mammalian lectin specific for mannose and *N*-acetylglucosamine. MBP is mainly synthesized in the liver and occurs naturally in two forms, serum MBP (S-MBP) and intracellular MBP (I-MBP). S-MBP activates complement in association with MBP-associated serine proteases via the lectin pathway. Despite our previous study (Mori, K., Kawasaki, T., and Yamashina, I. (1984) *Arch. Biochem. Biophys.* 232, 223-233), the subcellular localization of I-MBP and its

functional implication have not been clarified yet. Here, as an extension of our previous studies, we have demonstrated that the expression of human MBP cDNA reproduces native MBP differentiation of S-MBP and I-MBP in human hepatoma cells. I-MBP shows distinct accumulation in cytoplasmic granules, and is predominantly localized in the endoplasmic reticulum (ER) and involved in COPII vesicle-mediated ER-to-Golgi transport. However, the subcellular localization of either a mutant (C236S/C244S) I-MBP, which lacks carbohydrate-binding activity, or the wild-type I-MBP in tunicamycin-treated cells shows an equally diffuse cytoplasmic distribution, suggesting that the unique accumulation of I-MBP in the ER and COPII vesicles is mediated by an *N*-glycan-lectin interaction. Furthermore, the binding of I-MBP with glycoprotein intermediates occurs in the ER, which is carbohydrate- and pH-dependent, and is affected by glucose-trimmed high-mannose-type oligosaccharides. These results strongly indicate that I-MBP may function as a cargo transport lectin facilitating ER-to-Golgi traffic in glycoprotein quality control.

3.925 Stepwise proteolysis liberates tau fragments that nucleate the Alzheimer-like aggregation of full-length tau in a neuronal cell model
Wang, Y.P., Biernat, J., Pickjardt, M., Mandelkow, E. Amd Mandelkow, E-M.
*PNAS, **104(24)**, 10252-10257 (2007)*

Tau is a highly soluble protein, yet it aggregates abnormally in Alzheimer's disease. Here, we address the question of proteolytic processing of tau and the nucleation of aggregates by tau fragments. We show in neuronal cell models that fragments of the repeat domain of tau containing mutations of FTDP17 (frontotemporal dementia with parkinsonism linked to chromosome 17), produced by endogenous proteases, can induce the aggregation of full-length tau. Fragments are generated by successive cleavages, first N-terminally between K257 and S258, then C-terminally around residues 353–364; conversely, when the N-terminal cleavage is inhibited, no fragmentation and aggregation takes place. The C-terminal truncation and the coaggregation of fragments with full-length tau depends on the propensity for β-structure. The aggregation is modulated by phosphorylation but does not depend on it. Aggregation but not fragmentation as such is toxic to cells; conversely, toxicity can be prevented by inhibiting either aggregation or proteolysis. The results reveal a novel pathway of abnormal tau aggregation in neuronal cells.

3.926 Lipid Rafts Are Triage Centers for Multimeric and Monomeric Thyrotropin Receptor Regulation
Latif, R., Ando, T. and Davies, T.F.
*Endocrinology, **148(7)**, 3164-3175 (2007)*

The TSH receptor (TSHR), a heptahelical G protein-coupled receptor on the surface of thyrocytes, is a major autoantigen and physiological regulator of the thyroid gland. Unlike other G protein-coupled receptors, the TSHR undergoes posttranslational cleavage of its ectodomain, leading to the existence of several forms of the receptor on the plasma membrane. We previously hypothesized that to achieve high fidelity and specificity of TSH ligand or TSHR autoantibody signaling, the TSHR may compartmentalize into microdomains within the plasma membrane. In support of this hypothesis we have shown previously that TSHRs reside in GM_1 ganglioside-enriched lipid rafts in the plasma membrane of TSHR-expressing cells. In this study, we further explored the different forms of TSHRs that reside in lipid rafts. We studied both TSHR-transfected cells and rat thyrocytes, using both nondetergent biochemical analyses and receptor-lipid raft colocalization. Using the biochemical approach, we observed that monomeric receptors existed in both raft and nonraft fractions of the cell surface in the steady state. We also demonstrated that the multimeric forms of the receptor were preferentially partitioned into the lipid microdomains. Different TSHR forms, including multimers, were dynamically regulated both by receptor-specific and postreceptor-specific modulators. TSH ligand and TSHR antibody of the stimulating variety induced a decrease of multimeric forms in the raft fractions. In addition, multimeric and monomeric forms of the receptor were both associated with $G_{s\alpha}$ within and without the rafts. Although failure to achieve total lipid raft disruption prevented a conclusion regarding the relative power of TSHR signaling within and without the raft domains, these data showed clearly that not only were a significant proportion of TSHRs residing within lipid microdomains but that constitutive multimerization of TSHRs was actually regulated within the lipid rafts.

3.927 Correlation of Golgi localization of ZW10 and centrosomal accumulation of dynactin
Arasaki, K., Uemura, T., Tani, K. and Tagaya, M.
*Biochem. Biophys. Res. Comm., **359(3)**, 811-816 (2007)*

ZW10 participates in the termination of the spindle checkpoint during mitosis by interacting with dynamitin, a subunit of the dynein accessory complex dynactin. We previously showed that ZW10 is attached to the endoplasmic reticulum through RINT-1 in interphase HeLa cells and involved in membrane transport between the endoplasmic reticulum and Golgi. Although a recent study demonstrated that ZW10 is localized in the Golgi in COS7 cells, the mechanism that regulates ZW10 localization remains unknown. In this study we showed a correlation between the Golgi localization of ZW10 and the centrosomal accumulation of dynactin. The amounts of ZW10 associated with dynactin were larger in cells where ZW10 was present in the Golgi than those where ZW10 was not in the Golgi. The targeting of ZW10 to the perinuclear Golgi region was found to depend on the perinuclear accumulation of dynactin, suggesting that dynactin regulates ZW10 localization.

3.928 Siva is an apoptosis-selective p53 target gene important for neuronal cell death
Jacobs, S.B.R., Basak, S., Murray, J., Pathak, N. and Attardi, L.D.
*Cell Death and Differentiation, **14**, 1374-1385 (2007)*

p53 plays a central role in neuronal cell death resulting from acute injury or disease. To define the pathway by which p53 triggers apoptosis, we used microarray analysis to identify p53 target genes specifically upregulated during apoptosis but not cell cycle arrest. This analysis identified a small subset of targets highly selective for the p53 apoptotic response, including Siva, a proapoptotic protein whose function is not well understood. *Siva*'s expression pattern suggests that it plays an instructive role in apoptosis, and accordingly, we demonstrate that Siva is essential for p53-dependent apoptosis in cerebellar granule neurons. In addition, we determine that endogenous Siva is associated with the plasma membrane and that Caspase-8 and Bid are important for neuronal apoptosis. Our studies highlight the participation of membrane signaling events in p53's apoptotic program in primary neurons and have significant implications for understanding the mechanisms underlying pathogenesis after neuronal injury and in neurodegenerative diseases.

3.929 Identification of an ADAM2-ADAM3 Complex on the Surface of Mouse Testicular Germ Cells and Cauda Epididymal Sperm
Nishimura, H., Myles, D.G. and Primakoff, P.
*J. Biol. Chem., **282(24)**, 17900-17907 (2007)*

Male mice lacking ADAM2 (fertilin β) or ADAM3 (cyritestin) are infertile; cauda epididymal sperm (mature sperm) from these mutant mice cannot bind to the egg zona pellucida. ADAM3 is barely present in *Adam2*-null sperm, despite normal levels of this protein in *Adam2*-null testicular germ cells (TGCs; sperm precursor cells). Here, we have explored the molecular basis for the loss of ADAM3 in *Adam2*-null TGCs to clarify the biosynthetic and functional linkage of ADAM2 and ADAM3. A small portion of total ADAM3 was found present on the surface of wild-type and *Adam2*$^{-/-}$ TGCs at similar levels. In the *Adam2*-null TGCs, however, surface-localized ADAM3 exhibited an increased amount of an endoglycosidase H-resistant form that may be related to instability of ADAM3. Moreover, we found a complex between ADAM2 and ADAM3 on the surface of TGCs and sperm. The intracellular chaperone calnexin was a component of the testicular ADAM2-ADAM3 complex. Our findings suggest that the association with ADAM2 is a key element for stability of ADAM3 in epididymal sperm. The presence of the ADAM2-ADAM3 complex in sperm also suggests a potential role of ADAM2 with ADAM3 in sperm binding to the egg zona pellucida.

3.930 Productive Human Immunodeficiency Virus Type 1 Assembly Takes Place at the Plasma Membrane
Finzi, A., Orthwein, A., Mercier, J. and Cohen, E.A.
*J. Virol., **81(14)**, 7476-7490 (2007)*

Gag proteins are necessary and sufficient to direct human immunodeficiency virus type 1 (HIV-1) particle assembly and budding. Recent evidence suggests that Gag targeting to late endosomal/multivesicular body (LE/MVB) compartments occurs prior to viral particle budding at the plasma membrane (PM). However, the route that Gag follows before reaching its steady-state destinations still remains a subject of debate. Using a subcellular fractionation method that separates PM from LE/MVB combined with pulse-chase labeling, we analyzed Gag trafficking in HIV-1-producing HEK 293T cells. Our results reveal that the majority of newly synthesized Gag is primarily targeted to the PM. While PM-targeted Gag was efficiently released, a significant fraction of the remaining cell surface-associated Gag was found to be subsequently internalized to LE/MVB, where it accumulated, thus accounting for the majority of LE/MVB-associated Gag. Importantly, this accumulation of Gag in LE/MVB was found to be cholesterol dependent since it was

sensitive to the sterol-binding drugs filipin and methyl-ß-cyclodextrin. These results point towards the PM as being the primary site of productive HIV-1 assembly in cells that also support Gag accumulation in intracellular compartments.

3.931 Pitfalls in isolating lipid rafts
Nothdurfter, C., Rammes, G., Rein, T. and Rupprecht, R.
Nature review Neurosci., 8 567 (2007)

The recent Review by Allen *et al*. on lipid raft microdomains and neurotransmitter receptor signalling[1] provides an excellent overview of important structural and functional aspects of these specific membrane microdomains, with a particular focus on their role in the nervous system. Nevertheless, we would like to emphasize two important aspects. As outlined in the Review, the valid and reproducible isolation of lipid rafts is not trivial, but mandatory to draw correct conclusions. Inconsistencies in raft isolation procedures, for example the type, amount and duration of detergent use, make results difficult to compare and may contribute to some controversies in the field. A particularly important issue is the presentation of both a positive and a negative control. Mostly, caveolin 1 or flotillin 1 are used as raft marker proteins[2, 3, 4], whereas the transferrin receptor or other proteins such as the Na^+/K^+-ATPase are reported as non-raft proteins[5, 6, 7]. Only a few studies show convincing data of a clear separation of raft from non-raft proteins[5, 6, 8]. This separation relies highly on the preparation procedure, in particular the use of detergent, for which the type of detergent, the concentration and the duration of incubation are the main determinants. Furthermore, the preparation procedure should be chosen according to the type of tissue under investigation. An example from our laboratory, in which we isolated caveolin 1 and flotillin 1 as raft-associated proteins and the transferrin receptor as a non-raft protein, illustrates this need to vary conditions for different tissue types (Fig. 1). When investigating the concentration of detergent and incubation time needed to separate raft from non-raft proteins within the range reported in the literature[5, 6], we also observed a dose–response relationship[8]. Future studies should therefore clearly demonstrate that the method selected is appropriate for the tissue type under investigation to separate raft from non-raft proteins when claiming raft association of particular proteins.

3.932 Caspase-8 and c-FLIPL Associate in Lipid Rafts with NF-κB Adaptors during T Cell Activation
Misra, R.S. et al
J. Biol. Chem., ***282(27)***, *19365-19374 (2007)*

Humans and mice lacking functional caspase-8 in T cells manifest a profound immunodeficiency syndrome due to defective T cell antigen receptor (TCR)-induced NF-κB signaling and proliferation. It is unknown how caspase-8 is activated following T cell stimulation, and what is the caspase-8 substrate(s) that is necessary to initiate T cell cycling. We observe that following TCR ligation, a small portion of total cellular caspase-8 and c-$FLIP_L$ rapidly migrate to lipid rafts where they associate in an active caspase complex. Activation of caspase-8 in lipid rafts is followed by rapid cleavage of c-$FLIP_L$ at a known caspase-8 cleavage site. The active caspase·c-FLIP complex forms in the absence of Fas (CD95/APO1) and associates with the NF-κB signaling molecules RIP1, TRAF2, and TRAF6, as well as upstream NF-κB regulators PKCθ, CARMA1, Bcl-10, and MALT1, which connect to the TCR. The lack of caspase-8 results in the absence of MALT1 and Bcl-10 in the active caspase complex. Consistent with this observation, inhibition of caspase activity attenuates NF-κB activation. The current findings define a link among TCR, caspases, and the NF-κB pathway that occurs in a sequestered lipid raft environment in T cells.

3.933 Lack of a role of membrane-protein interactions in flow-dependent activation of EnaC
Carattino, M.D., Lin, W., Hill, W.G., Satlin, L.M. and Kleyman, T.R.
Am. J. Physiol. Renal Physiol., ***293,*** *F316-F324 (2007)*

Rates of Na^+ absorption in the distal nephron increase proportionally with the rates of tubular flow. We tested the hypothesis that the deformation or tension generated in the plasma membrane in response to flow activates the epithelial sodium channel (ENaC). We modified the physical properties of the membrane by changing the temperature and the content of cholesterol. Rates of net Na^+ absorption measured in cortical collecting ducts (CCDs) perfused at room temperature at slow (~1) and fast (~5 $nl \cdot min^{-1} \cdot mm^{-1}$) flow rates were less than those measured at 37°C at the same flow rates, although increases in tubular fluid flow rates led to comparable relative increases in net Na^+ absorption at both temperatures. *Xenopus laevis* oocytes expressing ENaC responded to an increase in shear stress at 22–25°C with a discrete delay followed by a monoexponential increase in whole-cell Na^+ currents. We observed that temperature affected *1*) basal

currents, *2*) delay times, *3*) kinetics of activation, and *4*) fold-increase in macroscopic currents in response to flow. The magnitude of the response to flow displayed biphasic behavior as a function of temperature, with a minimal value at 25°C. Steady-state fluorescence anisotropic measurements of purified plasma membranes did not show any obvious phase transition behavior over a temperature range from 8.3°C to 36.5°C. Modification of the content of membrane cholesterol did not affect the response to flow. Our results suggest that the flow-dependent activation of ENaC is not influenced by modifications in the intrinsic properties of the plasma membrane.

3.934 Ceramide transfer protein function is essential for normal oxidative stress response and lifespan
Rao, R.P. et al
*PNAS, **104(27)**, 11364-11369 (2007)*

Ceramide transfer protein (CERT) transfers ceramide from the endoplasmic reticulum to the Golgi complex, a process critical in synthesis and maintenance of normal levels of sphingolipids in mammalian cells. However, how its function is integrated into development and physiology of the animal is less clear. Here, we report the *in vivo* consequences of loss of functional CERT protein. We generated *Drosophila melanogaster* mutant flies lacking a functional CERT (Dcert) protein using chemical mutagenesis and a Western blot-based genetic screen. The mutant flies die early between days 10 and 30, whereas controls lived between 75 and 90 days. They display >70% decrease in ceramide phosphoethanolamine (the sphingomyelin analog in *Drosophila*) and ceramide. These changes resulted in increased plasma membrane fluidity that renders them susceptible to reactive oxygen species and results in enhanced oxidative damage to cellular proteins. Consequently, the flies showed reduced thermal tolerance that was exacerbated with aging and metabolic compromise such as decreasing ATP and increasing glucose levels, reminiscent of premature aging. Our studies demonstrate that maintenance of physiological levels of ceramide phosphoethanolamine by CERT *in vivo* is required to prevent oxidative damages to cellular components that are critical for viability and normal lifespan of the animal.

3.935 Regulatory Binding Partners and Complexes of NHE3
Donowitz, M. and Li, X.
*Physiol. Rev., **87**, 825-972 (2007)*

NHE3 is the brush-border (BB) Na^+/H^+ exchanger of small intestine, colon, and renal proximal tubule which is involved in large amounts of neutral Na^+ absorption. NHE3 is a highly regulated transporter, being both stimulated and inhibited by signaling that mimics the postprandial state. It also undergoes downregulation in diarrheal diseases as well as changes in renal disorders. For this regulation, NHE3 exists in large, multiprotein complexes in which it associates with at least nine other proteins. This review deals with short-term regulation of NHE3 and the identity and function of its recognized interacting partners and the multiprotein complexes in which NHE3 functions.

3.936 Filamin-A regulates actin-dependent clustering of HIV receptors
Jimenez-Baranda, S. et al
*Nature Cell Biol., **9(7)**, 838-846 (2007)*

Human immunodeficiency virus (HIV)-1 infection requires envelope (Env) glycoprotein gp120-induced clustering of CD4 and coreceptors (CCR5 or CXCR4) on the cell surface; this enables Env gp41 activation and formation of a complex that mediates fusion between Env-containing and target-cell membranes1. Kinetic studies show that viral receptors are actively transported to the Env-receptor interface in a process that depends on plasma membrane composition and the actin cytoskeleton2, 3, 4, 5, 6, 7. The mechanisms by which HIV-1 induces F-actin rearrangement in the target cell remain largely unknown. Here, we show that CD4 and the coreceptors interact with the actin-binding protein filamin-A, whose binding to HIV-1 receptors regulates their clustering on the cell surface. We found that gp120 binding to cell receptors induces transient cofilin-phosphorylation inactivation through a RhoA–ROCK-dependent mechanism. Blockade of filamin-A interaction with CD4 and/or coreceptors inhibits gp120-induced RhoA activation and cofilin inactivation. Our results thus identify filamin-A as an adaptor protein that links HIV-1 receptors to the actin cytoskeleton remodelling machinery, which may facilitate virus infection.

3.937 Cholesterol depletion alters detergent-specific solubility profiles of selected tight junction proteins and the phosphorylation of occluding
Lynch, R.D. et al

Exp. Cell Res., ***313(2)***, *2597-2610 (2007)*

Differential centrifugation of Triton X-100 or CHAPS lysates from control and cholesterol (CH)-depleted MDCK II cells, segregated integral tight junction (TJ) proteins associated with detergent-resistant membranes (DRMs) into two groups. Group A proteins (occludin, claudin-2 and -3) were detected in large, intermediate and small aggregates in both detergents, whereas group B proteins (claudin-1, -4 and -7) were observed in small aggregates in TX-100 and in intermediate and small aggregates in CHAPS. Depletion of CH altered the distribution of group A and B proteins among the three size categories in a detergent-specific manner. In lysates produced with octyl glucoside, a detergent that selectively extracts proteins from DRMs, group A proteins were undetectable in large aggregates and CH depletion did not alter the distribution of either group A or B proteins in intermediate or small aggregates. Neither occludin (group A) nor claudin-1 (group B) was in intimate enough contact with CH to be cross-linked to [^{3}H]-photo-cholesterol. However, antibodies to either TJ protein co-immunoprecipitated caveolin-1, a CH-binding protein. Unlike claudins, occludin's presence in TJs and DRMs did not require palmitoylation. Equilibrium density centrifugation on discontinuous OptiPrep gradients revealed detergent-related differences in the densities of TJ-bearing DRMs. There was little or no change in those densities after CH depletion. Removing CH from the plasma membrane increased tyrosine and threonine phosphorylation of occludin, and transepithelial electrical resistance (TER) within 30 min. After 2 h of CH efflux, phospho-occludin levels and TER fell below control values. We conclude that the association of integral TJ proteins with DRMS, pelleted at low speeds, is partially CH-dependent. However, the buoyant density of TJ-associated DRMs is a function of the detergent used and is insensitive to decreases in CH.

3.938

3.939 **The Pseudomonas aeruginosa Secreted Protein PA2934 Decreases Apical Membrane Expression of the Cystic Fibrosis Transmembrane Conductance Regulator**
MacEachran, D.P. et al
Infect. Immun., ***75(8)***, *3902-3912 (2007)*

We previously reported that *Pseudomonas aeruginosa* PA14 secretes a protein that can reduce the apical membrane expression of the *c*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator (CFTR) protein. Here we report that we have used a proteomic approach to identify this secreted protein as PA2394, and we have named the gene *cif*, for *C*FTR *i*nhibitory *f*actor. We demonstrate that Cif is a secreted protein and is found associated with outer membrane-derived vesicles. Expression of Cif in *Escherichia coli* and purification of the C-terminal six-His-tagged Cif protein showed that Cif is necessary and sufficient to mediate the reduction in apical membrane expression of CFTR and a concomitant reduction in CFTR-mediated Cl^- ion secretion. Cif demonstrates epoxide hydrolase activity in vitro and requires a highly conserved histidine residue identified in α/ß hydrolase family enzymes to catalyze this reaction. Mutating this histidine residue also abolishes the ability of Cif to reduce apical membrane CFTR expression. Finally, we demonstrate that the *cif* gene is expressed in the cystic fibrosis (CF) lung and that nonmucoid isolates of *P. aeruginosa* show greater expression of the gene than do mucoid isolates. We propose a model in which the Cif-mediated decrease in apical membrane expression of CFTR by environmental isolates of *P. aeruginosa* facilitates the colonization of the CF lung by this microbe.

3.940 **Caveolin-associated Accumulation of Globotriaosylceramide in the Vascular Endothelium of α-Galactosidase A Null Mice**
Shu, L. and Shayman, J.A.
J. Biol. Chem., ***282(29)***, *20960-20967 (2007)*

Cardiovascular complications, including stroke and myocardial infarction, result in premature mortality in patients with Fabry disease, an X-linked deficiency of α-galactosidase A (α-Gal A). The enzymatic defect results in the deposition of globotriaosylceramide (Gb3) in the vascular endothelium. To better understand the underlying pathogenesis of Fabry disease, the caveolar lipid content of primary cultured mouse aortic endothelial cells isolated from α-Gal A null mice was measured. Lipid mass analysis revealed that the excessive Gb3 in cultured α-*Gal A*-deficient mouse aortic endothelial cells accumulated in endothelial plasma membrane caveolar fractions. The levels of glucosylceramide and lactosylceramide increased in parallel with Gb3 levels in an age-dependent manner, whereas globotetraosylceramide (Gb4) levels reached maximal levels by 6 months of age and then rapidly decreased at older ages. The levels of

cholesterol enriched in caveolar membranes declined in parallel with the progressive deposition of Gb3. Depleting Gb3 with recombinant human α-Gal A protein or D-*threo*-ethylenedioxyphenyl-P4, an inhibitor of glucosylceramide synthase, restored cholesterol in cultured α-Gal A-deficient mouse aortic endothelial cell caveolae. By contrast, recombinant human α-Gal A was less effective in normalizing the cholesterol content. These results demonstrate the caveolar accumulation of glycosphingolipids in an *in vitro* model of a lysosomal storage disease and raise the possibility that dynamic changes in the composition of plasma membrane lipid microdomains may mediate the endothelial dysfunction seen in Fabry disease.

3.941 Lipid Rafts Establish Calcium Waves in Hepatocytes
Nagata, J. et al
*Gastroenterology, **133(1)**, 256-267 (2007)*

Background & Aims: Polarity is critical for hepatocyte function. Ca^{2+} waves are polarized in hepatocytes because the inositol 1,4,5-trisphosphate receptor (InsP3R) is concentrated in the pericanalicular region, but the basis for this localization is unknown. We examined whether pericanalicular localization of the InsP3R and its action to trigger Ca^{2+} waves depends on lipid rafts. Methods: Experiments were performed using isolated rat hepatocyte couplets and pancreatic acini, plus SkHep1 cells as nonpolarized controls. The cholesterol depleting agent methyl-beta-cyclodextrin (mβCD) was used to disrupt lipid rafts. InsP3R isoforms were examined by immunoblot and immunofluorescence. Ca^{2+} waves were examined by confocal microscopy. *Results:* Type II InsP3Rs initially were localized to only some endoplasmic reticulum fractions in hepatocytes, but redistributed into all fractions in mβCD-treated cells. This InsP3R isoform was concentrated in the pericanalicular region, but redistributed throughout the cell after mβCD treatment. Vasopressin-induced Ca^{2+} signals began as apical-to-basal Ca^{2+} waves, and mβCD slowed the wave speed and prolonged the rise time. MβCD had a similar effect on Ca^{2+} waves in acinar cells but did not affect Ca^{2+} signals in SkHep1 cells, suggesting that cholesterol depletion has similar effects among polarized epithelia, but this is not a nonspecific effect of mβCD. *Conclusions:* Lipid rafts are responsible for the pericanalicular accumulation of InsP3R in hepatocytes, and for the polarized Ca^{2+} waves that result. Signaling microdomains exist not only in the plasma membrane, but also in the nearby endoplasmic reticulum, which in turn, helps establish and maintain structural and functional polarity.

3.942 Alzheimer's presenilin 1 modulates sorting of APP and its carboxyl-terminal fragments in cerebral neurons in vivo
Gandy, S. et al
*J. Neurochem., **102**, 619-626 (2007)*

Studies in continuously cultured cells have established that familial Alzheimer's disease (FAD) mutant presenilin 1 (PS1) delays exit of the amyloid precursor protein (APP) from the *trans*-Golgi network (TGN). Here we report the first description of PS1-regulated APP trafficking in cerebral neurons in culture and *in vivo*. Using neurons from transgenic mice or a cell-free APP transport vesicle biogenesis system derived from the TGN of those neurons, we demonstrated that knocking-in an FAD-associated mutant PS1 transgene was associated with delayed kinetics of APP arrival at the cell surface. Apparently, this delay was at least partially attributable to impaired exit of APP from the TGN, which was documented in the cell-free APP transport vesicle biogenesis assay. To extend the study to APP and carboxyl terminal fragment (CTF) trafficking to cerebral neurons *in vivo*, we performed subcellular fractionation of brains from APP transgenic mice, some of which carried a second transgene encoding an FAD-associated mutant form of PS1. The presence of the FAD mutant PS1 was associated with a slight shift in the subcellular localization of both holoAPP and APP CTFs toward iodixanol density gradient fractions that were enriched in a marker for the TGN. In a parallel set of experiments, we used an APP : furin chimeric protein strategy to test the effect of artificially forcing TGN concentration of an APP : furin chimera that could be a substrate for β- and γ-cleavage. This chimeric substrate generated excess Aβ42 when compared with wildtype APP. These data indicate that the presence of an FAD-associated mutant human PS1 transgene is associated with redistribution of the APP and APP CTFs in brain neurons toward TGN-enriched fractions. The chimera experiment suggests that TGN-enrichment of a β-/γ-secretase substrate may play an integral role in the action of mutant PS1 to elevate brain levels of Aβ42.

3.943 Probing the Membrane Environment of the TOR Kinases Reveals Functional Interactions between TORC1, Actin, and Membrane Trafficking in Saccharomyces cerevisiae
Aronova, S., wedaman, K., Anderson, S., Yates III, J. and Powers, T.
*Mol. Biol. Cell, **18**, 2779-2794 (2007)*

The TOR kinases are regulators of growth in eukaryotic cells that assemble into two distinct protein complexes, TORC1 and TORC2, where TORC1 is inhibited by the antibiotic rapamycin. Present models favor a view wherein TORC1 regulates cell mass accumulation, and TORC2 regulates spatial aspects of growth, including organization of the actin cytoskeleton. Here, we demonstrate that in yeast both TORC1 and TORC2 fractionate with a novel form of detergent-resistant membranes that are distinct from detergent-resistant plasma membrane "rafts." Proteomic analysis of these TOR-associated membranes revealed the presence of regulators of endocytosis and the actin cytoskeleton. Genetic analyses revealed a significant number of interactions between these components and TORC1, demonstrating a functional link between TORC1 and actin/endocytosis-related genes. Moreover, we found that inhibition of TORC1 by rapamycin 1) disrupted actin polarization, 2) delayed actin repolarization after glucose starvation, and 3) delayed accumulation of lucifer yellow within the vacuole. By combining our genetic results with database mining, we constructed a map of interactions that led to the identification of additional genetic interactions between TORC1 and components involved in membrane trafficking. Together, these results reveal the broad scope of cellular processes influenced by TORC1, and they underscore the functional overlap between TORC1 and TORC2.

3.944 Evidence for Coupled Biogenesis of Yeast Gap1 Permease and Sphingolipids: Essential Role in Transport Activity and Normal Control by Ubiquitination
Lauwers, E., Grossmann, G. and Andre, B.
*Mol. Biol. Cell, **18**, 3068-3080 (2007)*

Current models for plasma membrane organization integrate the emerging concepts that membrane proteins tightly associate with surrounding lipids and that biogenesis of surface proteins and lipids may be coupled. We show here that the yeast general amino acid permease Gap1 synthesized in the absence of sphingolipid (SL) biosynthesis is delivered to the cell surface but undergoes rapid and unregulated down-regulation. Furthermore, the permease produced under these conditions but blocked at the cell surface is inactive, soluble in detergent, and more sensitive to proteases. We also show that SL biogenesis is crucial during Gap1 production and secretion but that it is dispensable once Gap1 has reached the plasma membrane. Moreover, the defects displayed by cell surface Gap1 neosynthesized in the absence of SL biosynthesis are not compensated by subsequent restoration of SL production. Finally, we show that down-regulation of Gap1 caused by lack of SL biogenesis involves the ubiquitination of the protein on lysines normally not accessible to ubiquitination and close to the membrane. We propose that coupled biogenesis of Gap1 and SLs would create an SL microenvironment essential to the normal conformation, function, and control of ubiquitination of the permease.

3.945 Naked2 Acts as a Cargo Recognition and Targeting Protein to Ensure Proper Delivery and Fusion of TGF-α–containing Exocytic Vesicles at the Lower Lateral Membrane of Polarized MDCK Cells
Li, C. et al
*Mol. Biol. Cell, **18**, 3081-3093 (2007)*

Transforming growth factor-α (TGF-α) is the major autocrine EGF receptor ligand in vivo. In polarized epithelial cells, proTGF-α is synthesized and then delivered to the basolateral cell surface. We previously reported that Naked2 interacts with basolateral sorting determinants in the cytoplasmic tail of a Golgi-processed form of TGF-α and that TGF-α is not detected at the basolateral surface of Madin-Darby canine kidney (MDCK) cells expressing myristoylation-deficient (G2A) Naked2. By high-resolution microscopy, we now show that wild-type, but not G2A, Naked2-associated vesicles fuse at the plasma membrane. We further demonstrate that Naked2-associated vesicles are delivered to the lower lateral membrane of polarized MDCK cells independent of μ1B adaptin. We identify a basolateral targeting segment within Naked2; residues 1-173 redirect NHERF-1 from the apical cytoplasm to the basolateral membrane, and internal deletion of residues 37-104 results in apical mislocalization of Naked2 and TGF-α. Short hairpin RNA knockdown of Naked2 leads to a dramatic reduction in the 16-kDa cell surface isoform of TGF-α and increased cytosolic TGF-α immunoreactivity. We propose that Naked2 acts as a cargo recognition and targeting (CaRT) protein to ensure proper delivery, tethering, and fusion of TGF-α–containing vesicles to a distinct region at the basolateral surface of polarized epithelial cells.

3.946 Characterization of the Properties and Trafficking of an Anchorless Form of the Prion Protein
Campana, V. et al
*J. Biol. Chem., **282(31)**, 22747-22756 (2007)*

Conversion of PrP^{C} into PrP^{Sc} is the central event in the pathogenesis of transmissible prion diseases. Although the molecular basis of this event and the intracellular compartment where it occurs are not yet understood, the association of PrP with cellular membranes and in particular its presence in detergent-resistant microdomains appears to be of critical importance. In addition it appears that scrapie conversion requires membrane-bound glycosylphosphatidylinositol (GPI)-linked PrP. The GPI anchor may affect either the conformation, the intracellular localization, or the association of the prion protein with specific membrane domains. However, how this occurs is not known. To understand the relevance of the GPI anchor for the cellular behavior of PrP, we have studied the biosynthesis and localization of a PrP version which lacks the GPI anchor attachment signal (PrPΔGPI). We found that PrPΔGPI is tethered to cell membranes and associates to membrane detergent-resistant microdomains but does not assume a transmembrane topology. Differently to PrP^{C}, this protein does not localize at the cell surface but is mainly released in the culture media in a fully glycosylated soluble form. The cellular behavior of anchorless PrP explains why PrPΔGPI Tg mice can be infected but do not show the classical signs of the disorder, thus indicating that the plasma membrane localization of PrP^{C} and/or of the converted scrapie form might be necessary for the development of a symptomatic disease.

3.947 The SUMO Conjugating Enzyme Ubc9 is a Regulator of GLUT4 Turnover and Targeting to the Insulin-Responsive Storage Compartment in 3T3-L1 Adipocytes
Liu, L-B., Omatra, W., Kojima, I. and Shibata, H.
*Diabetes, **56**, 1977-1985 (2007)*

The small ubiquitin-related modifier (SUMO) conjugating enzyme Ubc9 has been shown to upregulate GLUT4 in L6 myoblast cells, although the mechanism of action has remained undefined. Here we investigated the physiological significance of Ubc9 in GLUT4 turnover and subcellular targeting by adenovirus vector–mediated overexpression and by small interfering RNA (siRNA)-mediated gene silencing of Ubc9 in 3T3-L1 adipocytes. Overexpression of Ubc9 resulted in an inhibition of GLUT4 degradation and promoted its targeting to the unique insulin-responsive GLUT4 storage compartment (GSC), leading to an increase in GLUT4 amount and insulin-responsive glucose transport in 3T3-L1 adipocytes. Overexpression of Ubc9 also antagonized GLUT4 downregulation and its selective loss in GSC induced by long-term insulin stimulation. By contrast, siRNA-mediated depletion of Ubc9 accelerated GLUT4 degradation and decreased the amount of the transporter, concurrent with its selective loss in GSC, which resulted in attenuated insulin-responsive glucose transport. Intriguingly, overexpression of the catalytically inactive mutant Ubc9-C93A produced effects indistinguishable from those with wild-type Ubc9, suggesting that Ubc9 regulates GLUT4 turnover and targeting to GSC by a mechanism independent of its catalytic activity. Thus, Ubc9 is a pivotal regulator of the insulin sensitivity of glucose transport in adipocytes.

3.948 Core Protein Machinery for Mammalian Phosphatidylinositol 3,5-Bisphosphate Synthesis and Turnover That Regulates the Progression of Endosomal Transport: NOVEL SAC PHOSPHATASE JOINS THE ArPIKfyve-PIKfyve COMPLEX
Sbrissa, D. et al
*J. Biol. Chem., **282(33)**, 23878-23891 (2007)*

Perturbations in phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2)-synthesizing enzymes result in enlarged endocytic organelles from yeast to humans, indicating evolutionarily conserved function of PtdIns(3,5)P_2 in endosome-related events. This is reinforced by the structural and functional homology of yeast Vac14 and human Vac14 (ArPIKfyve), which activate yeast and mammalian PtdIns(3,5)P_2-producing enzymes, Fab1 and PIKfyve, respectively. In yeast, PtdIns(3,5)P_2-specific phosphatase, Fig4, in association with Vac14, turns over PtdIns(3,5)P_2, but whether such a mechanism operates in mammalian cells and what the identity of mammalian Fig4 may be are unknown. Here we have identified and characterized Sac3, a Sac domain phosphatase, as the Fig4 mammalian counterpart. Endogenous Sac3, a widespread 97-kDa protein, formed a stable ternary complex with ArPIKfyve and PIKfyve. Concordantly, Sac3 cofractionated and colocalized with ArPIKfyve and PIKfyve. The intrinsic $Sac3^{WT}$ phosphatase activity preferably hydrolyzed PtdIns(3,5)P_2 *in vitro*, although the other D5-phosphorylated polyphosphoinositides were also substrates. Ablation of endogenous Sac3 by short interfering RNAs elevated PtdIns(3,5)P_2 in ^{32}P-labeled HEK293 cells. Ectopically expressed $Sac3^{WT}$ in COS cells colocalized with and dilated EEA1-positive endosomes, consistent with the PtdIns(3,5)P_2 requirement in early endosome dynamics. *In vitro* reconstitution of carrier vesicle formation from donor early endosomes revealed a gain of function upon Sac3 loss, whereas PIKfyve or ArPIKfyve protein depletion produced a loss of function. These data demonstrate a coupling between the machinery for PtdIns(3,5)P_2 synthesis and

turnover achieved through a physical assembly of PIKfyve, ArPIKfyve, and Sac3. We suggest that the tight regulation in PtdIns(3,5)P_2 homeostasis is mechanistically linked to early endosome dynamics in the course of cargo transport.

3.949 Catalase Takes Part in Rat Liver Mitochondria Oxidative Stress Defense
Salvi, M. et al
J. Biol. Chem., ***282(33)***, *24407-24451 (2007)*

Highly purified rat liver mitochondria (RLM) when exposed to *tert*-butylhydroperoxide undergo matrix swelling, membrane potential collapse, and oxidation of glutathione and pyridine nucleotides, all events attributable to the induction of mitochondrial permeability transition. Instead, RLM, if treated with the same or higher amounts of H_2O_2 or tyramine, are insensitive or only partially sensitive, respectively, to mitochondrial permeability transition. In addition, the block of respiration by antimycin A added to RLM respiring in state 4 conditions, or the addition of H_2O_2, results in O_2 generation, which is blocked by the catalase inhibitors aminotriazole or KCN. In this regard, H_2O_2 decomposition yields molecular oxygen in a 2:1 stoichiometry, consistent with a catalatic mechanism with a rate constant of 0.0346 s^{-1}. The rate of H_2O_2 consumption is not influenced by respiratory substrates, succinate or glutamate-malate, nor by *N*-ethylmaleimide, suggesting that cytochrome *c* oxidase and the glutathione-glutathione peroxidase system are not significantly involved in this process. Instead, H_2O_2 consumption is considerably inhibited by KCN or aminotriazole, indicating activity by a hemoprotein. All these observations are compatible with the presence of endogenous heme-containing catalase with an activity of 825 ± 15 units, which contributes to mitochondrial protection against endogenous or exogenous H_2O_2. Mitochondrial catalase in liver most probably represents regulatory control of bioenergetic metabolism, but it may also be proposed for new therapeutic strategies against liver diseases. The constitutive presence of catalase inside mitochondria is demonstrated by several methodological approaches as follows: biochemical fractionating, proteinase K sensitivity, and immunogold electron microscopy on isolated RLM and whole rat liver tissue.

3.950 Cell-Surface Thioredoxin-1: Possible Involvement in Thiol-Mediated Leukocyte-Endothelial Cell Interaction Through Lipid Rafts
Hara, T. et al
Antioxidants & Redox Signaling, ***9(9)***, *1427-1437 (2007)*

Human thioredoxin-1 (hTrx) exhibits a disulfide reducing activity and was originally identified as a soluble cytokine-like factor secreted from cells of a human T-cell leukemia virus type I (HTLV-I)-transformed cell line. Recent studies have revealed that endogenous Trx plays an important role in cytoprotection against various oxidative stress–associated disorders. However, the function of exogenous Trx is still not fully understood. We report here that a cysteine-modified mutant of recombinant human Trx (rhTrx-C35S) binds to human umbilical vein endothelial cells (HUVECs) as well as stimulated T cells and rapidly enters these cells *via* lipid rafts. In addition, we found that endogenous Trx is expressed on the surface of HUVECs, including lipid rafts. These events suggest cell-surface Trx as a possible target of rhTrx-C35S. Furthermore, we found that anti-human Trx mouse monoclonal antibody inhibits adherence of LPS-stimulated human peripheral blood polymorphonuclear cells (PMNs) to HUVECs. This adherence was also suppressed by a recombinant human Trx (rhTrx), but not by a mutant rhTrx (rhTrx-C32S/C35S) with no reducing activity. Cell-surface Trx may be involved in the process of interaction between PMNs and HUVECs and a possible target of cysteine-modified exogenous Trx as well as wild-type exogenous Trx through redox regulation.

3.951 Lipid Raft–Mediated Uptake of Cysteine-Modified Thioredoxin-1: Apoptosis Enhancement by Inhibiting the Endogenous Thioredoxin-1
Kondo, N. et al
Antioxidants & Redox Signaling, ***9(9)***, *1439-1448 (2007)*

Thioredoxin-1 (TRX) plays important roles in cellular signaling by controlling the redox state of cysteine residues in target proteins. TRX is released in response to oxidative stress and shows various biologic functions from the extracellular environment. However, the mechanism by which extracellular TRX transduces the signal into the cells remains unclear. Here we report that the cysteine modification at the active site of TRX promotes the internalization of TRX into the cells. TRX-C35S, in which the cysteine at residue 35 of the active site was replaced with serine, was internalized more effectively than wild-type TRX in human T-cell leukemia virus–transformed T cells. TRX-C35S bound rapidly to the cell surface and was internalized into the cells dependent on lipid rafts in the plasma membrane. This process was

inhibited by wild-type TRX, reducing reagents such as dithiothreitol, and methyl-β-cyclodextrin, which disrupts lipid rafts. Moreover, the internalized TRX-C35S binds to endogenous TRX, resulting in the generation of intracellular reactive oxygen species (ROS) and enhanced *cis*-diamine-dichloroplatinum (II) (CDDP)-induced apoptosis *via* a ROS-mediated pathway involving apoptosis signal-regulating kinase-1 (ASK-1) activation. These findings suggest that the cysteine at the active site of TRX plays a key role in the internalization and signal transduction of extracellular TRX into the cells.

3.952 P-Glycoprotein is not present in mitochondrial membranes
Paterson, J.K. and Gottesman, M.M.
*Exp. Cell Res., **313(14)**, 3100-3103 (2007)*

Recent reports have indicated the presence of P-glycoprotein in crude mitochondrial membrane fractions, leading to the assumption that P-glycoprotein is present in mitochondrial membranes, and may be involved in transport across these membranes. To determine the validity of this claim, two cell lines overexpressing endogenous P-glycoprotein were investigated. Using various centrifugation steps, mitochondria were purified from these cells and analyzed by Western blot reaction with the anti-P-glycoprotein antibody C219 and organelle-specific antibodies. While P-glycoprotein is present in crude mitochondrial fractions, these fractions are contaminated with plasma membranes. Further purification of the mitochondria to remove plasma membranes revealed that P-glycoprotein is not expressed in mitochondria of the KB-V1 (vinblastine-resistant KB-3-1 cells) or MCF-7^{ADR} (adriamycin-resistant MCF-7 cells) cell lines. To further substantiate these findings, we used confocal microscopy and the anti-P-glycoprotein antibody 17F9. This demonstrated that in intact cells, P-glycoprotein is not present in mitochondria and is primarily localized to the plasma membrane. These findings are consistent with the role of P-glycoprotein in conferring multidrug resistance by decreasing cellular drug accumulation. Therefore, contrary to previous speculation, P-glycoprotein does not confer cellular protection by residing in mitochondrial membranes.

3.953 Ubiquitination of Human Immunodeficiency Virus Type 1 Gag Is Highly Dependent on Gag Membrane Association
Jäger, S., Gottwein, E. and Kräusslich, H-G.
*J. Virol., **81(17)**, 9193-9201 (2007)*

Ubiquitin is important for the release of human immunodeficiency virus 1 (HIV-1) and several other retroviruses. All major domains of the HIV-1 Gag protein are monoubiquitinated, but the modifying machinery and the function of HIV-1 Gag ubiquitination remain unclear. Here, we show that the induction of a late budding arrest by mutation of the HIV-1 PTAP motif or by specific inhibition of selected ESCRT components leads to an increase of Gag-ubiquitin conjugates in cells, which coincides with an accumulation of detergent-insoluble, multimerized Gag at the plasma membrane. Membrane flotation experiments revealed that ubiquitinated Gag is highly enriched in membrane-bound fractions. Based on these findings, we propose that a blocking of virus release results in increased Gag ubiquitination as a consequence of its prolonged membrane association. Consistent with this, ubiquitination of a membrane-binding-defective (G2A)Gag mutant was dramatically reduced and the ubiquitination levels of truncated Gag proteins correlated with their abilities to bind to membranes. We therefore propose that membrane association and multimerization of HIV-1 Gag proteins, rather than a specific motif within Gag, trigger recognition by the cellular ubiquitination machinery.

3.954 Organellar proteomics to create the cell map
Au, C.E. et al
*Current Opinion in Cell Biology, **19**, 376-385 (2007)*

The elucidation of a complete, accurate, and permanent representation of the proteome of the mammalian cell may be achievable piecemeal by an organellar based approach. The small volume of organelles assures high protein concentrations. Providing isolated organelles are homogenous, this assures reliable protein characterization within the sensitivity and dynamic range limits of current mass spec based analysis. The stochastic aspect of peptide selection by tandem mass spectrometry for sequence determination by fragmentation is dealt with by multiple biological replicates as well as by prior protein separation on 1-D gels. Applications of this methodology to isolated synaptic vesicles, clathrin coated vesicles, endosomes, phagosomes, endoplasmic reticulum, and Golgi apparatus, as well as Golgi-derived COPI vesicles, have led to mechanistic insight into the identity and function of these organelles.

3.955 Cholesterol-Dependent and -Independent CD40 Internalization and Signaling Activation in Cardiovascular Endothelial Cells

Chen, J., Chen, L., Wang, G. and Tang, H.

*Artioscler. Thtomb. Vasc. Biol., **27**, 2005-2013 (2007)*

***Objective*—** It remains elusive how CD40 endocytosis or clustering on the cell surface is induced by different forms of CD40 agonist. This study aims to investigate whether lipid rafts differentially regulate CD40 traffic and signaling in proinflammatory activation of cardiovascular endothelial cells (ECs).
***Methods and Results*—** Using fluorescent microscopy and flow cytometry, we demonstrated that soluble CD40L and agonistic antibody G28.5 induced CD40 internalization via clathrin-independent pathway. Furthermore, depletion of cholesterol by methyl-ß-cyclodextrin (MCD) or siRNA knockdown of caveolin-1 efficiently blocked CD40 internalization, suggesting that caveolae-rafts pathway regulates CD40 internalization. In contrast, a membrane-bound CD40L mimic (megamer) triggered aggregation of CD40 rafts outside of the conventional cholera toxin B subunit-positive lipid rafts resistant to cholesterol depletion. Finally, both G28.5 and megamer induced CD40 translocation to Brij58-insoluble, low buoyant density rafts, a movement insensitive to cholesterol depletion. However, MCD effectively inhibited G28.5 but not megamer-induced CD40 activation, and such inhibition could be alleviated by cholesterol reconstitution, suggesting that 2 different raft structures of CD40 induced by G28.5 or megamer possess differential sensitivity to cellular cholesterol levels in downstream signaling.
***Conclusions*—** Depending on different forms of agonist, CD40 uses either a cholesterol-dependent or -independent mode for trafficking and signaling in ECs.
Although activated CD40 can translocate to lipid rafts despite cholesterol depletion, different forms of CD40L, either soluble or membrane-bound, required distinct membrane constituents and microdomains for CD40 internalization and signaling activation. In particular, stimulation with G28.5, but not membrane-bound CD40L, required cellular cholesterol for CD40 internalization and downstream signaling.

3.956 Localization of the Mouse 5-Hydroxytryptamine1A Receptor in Lipid Microdomains Depends on Its Palmitoylation and Is Involved in Receptor-Mediated Signaling

Renner, U. et al

*Mol. Pharmacol., **72(3)**, 502-513 (2007)*

In the present study, we have used wild-type and palmitoylation-deficient mouse 5-hydroxytryptamine$_{1A}$ receptor (5-HT1A) receptors fused to the yellow fluorescent protein- and the cyan fluorescent protein (CFP)-tagged α_{i3} subunit of heterotrimeric G-protein to study spatiotemporal distribution of the 5-HT1A-mediated signaling in living cells. We also addressed the question on the molecular mechanisms by which receptor palmitoylation may regulate communication between receptors and G_i-proteins. Our data demonstrate that activation of the 5-HT1A receptor caused a partial release of $G\alpha_i$ protein into the cytoplasm and that this translocation is accompanied by a significant increase of the intracellular Ca^{2+} concentration. In contrast, acylation-deficient 5-HT1A mutants failed to reproduce both $G\alpha_{i3}$-CFP relocation and changes in $[Ca^{2+}]_i$ upon agonist stimulation. By using gradient centrifugation and copatching assays, we also demonstrate that a significant fraction of the 5-HT1A receptor resides in membrane rafts, whereas the yield of the palmitoylation-deficient receptor in these membrane microdomains is reduced considerably. Our results suggest that receptor palmitoylation serves as a targeting signal responsible for the retention of the 5-HT1A receptor in membrane rafts. More importantly, the raft localization of the 5-HT1A receptor seems to be involved in receptor-mediated signaling.

3.957 Activated Ezrin Promotes Cell Migration through Recruitment of the GEF Dbl to Lipid Rafts and Preferential Downstream Activation of Cdc42

Prag, S. et al

*Mol. Biol. Cell, **18**, 2935-2948 (2007)*

Establishment of polarized cell morphology is a critical factor for migration and requires precise spatial and temporal activation of the Rho GTPases. Here, we describe a novel role of the actin-binding ezrin/radixin/moesin (ERM)-protein ezrin to be involved in recruiting Cdc42, but not Rac1, to lipid raft microdomains, as well as the subsequent activation of this Rho GTPase and the downstream effector p21-activated kinase (PAK)1, as shown by fluorescence lifetime imaging microscopy. The establishment of a leading plasma membrane and the polarized morphology necessary for random migration are also dependent on ERM function and Cdc42 in motile breast carcinoma cells. Mechanistically, we show that the recruitment of the ERM-interacting Rho/Cdc42-specific guanine nucleotide exchange factor Dbl to the plasma membrane and to lipid raft microdomains requires the phosphorylated, active conformer of ezrin,

which serves to tether the plasma membrane or its subdomains to the cytoskeleton. Together these data suggest a mechanism whereby precise spatial guanine nucleotide exchange of Cdc42 by Dbl is dependent on functional ERM proteins and is important for directional cell migration.

3.958 Human Papillomavirus Type 31 Uses a Caveolin 1- and Dynamin 2-Mediated Entry Pathway for Infection of Human Keratinocytes
Smith, J.L., Campos, S.K. and Ozbun, M.A.
J. Virol., ***81(18)****, 9922-9931 (2007)*

Papillomaviruses are species-specific and epitheliotropic DNA viruses that cause tumors in their natural hosts. Certain infections with genital human papillomavirus (HPV) types are causally related to cervical cancer development. Most papillomaviruses are thought to infect cells via a clathrin-dependent pathway, yet no studies have determined the entry route in permissive host epithelial cells. Employing fluorescently labeled and native virions, we tested the effects of dominant-negative and biochemical inhibitors of cellular endocytosis pathways. Infections of human keratinocytes, a natural host cell type for HPVs, were assessed visually and by infectious entry assays. We found that HPV type 31 (HPV31) entry and initiation of early infection events require both caveolin 1 and dynamin 2 and occur independently of clathrin-mediated endocytosis. Treatment with chlorpromazine and filipin had opposing effects on HPV31 and HPV16 infection. HPV31 entry was remarkably slow, with a half-time of ≈14 h, whereas the entry half-time of HPV16 was 4 h. Consistent with a caveola-mediated entry pathway for HPV31, the virions associated with detergent-resistant lipid rafts. During a 16-h microscopic tracking of HPV31 and HPV16 virions, no colocalization of the two viral types was observed. These data suggest that HPV31 and HPV16 virions use distinct routes for host epithelial cell entry.

3.959 Detergent resistance as a tool in membrane research
Lingwood, D. and Simons, K.
Nature Protocols, ***2(9)****, 2159-2165 (2007)*

The biological membrane is a complicated matrix wherein different lipid environments are thought to exist. The more ordered or raft environment has been perceived biochemically accessible via its relative resistance to detergent. This paper outlines the protocols developed in our laboratory for the analysis of such detergent-resistant membranes (DRMs). We stress the fact that DRMs are artifactual in nature and should not be equivocated to lipid rafts, their usefulness being limited to assigning raft-association potential most convincingly when changes in DRM composition are induced by biochemically/physiologically relevant events. These protocols are completed in 1–2 d.

3.960 Light-induced recruitment of INAD-signaling complexes to detergent-resistant lipid rafts in Drosophila photoreceptors
Sanxaridis, P.D. et al
Mol. Cell. Neurosci., ***36(1)****, 36-46 (2007)*

Here, we reveal a novel feature of the dynamic organization of signaling components in *Drosophila* photoreceptors. We show that the multi-PDZ protein INAD and its target proteins undergo light-induced recruitment to detergent-resistant membrane (DRM) rafts. Reduction of ergosterol, considered to be a key component of lipid rafts in *Drosophila*, resulted in a loss of INAD-signaling complexes associated with DRM fractions. Genetic analysis demonstrated that translocation of INAD-signaling complexes to DRM rafts requires activation of the entire phototransduction cascade, while constitutive activation of the light-activated channels resulted in recruitment of complexes to DRM rafts in the dark. Mutations affecting INAD and TRP showed that PDZ4 and PDZ5 domains of INAD, as well as the INAD–TRP interaction, are required for translocation of components to DRM rafts. Finally, selective recruitment of phosphorylated, and therefore activatable, eye-PKC to DRM rafts suggests that DRM domains are likely to function in signaling, rather than trafficking.

3.961 Involvement of cell surface ATP synthase in flow-induced ATP release by vascular endothelial cells
Yamamoto, K. et al
*Am. J. Physiol. Heart Circ. Physiol.,****293,*** *H1646-H1653 (2007)*

Endothelial cells (ECs) release ATP in response to shear stress, a mechanical force generated by blood flow, and the ATP released modulates EC functions through activation of purinoceptors. The molecular mechanism of the shear stress-induced ATP release, however, has not been fully elucidated. In this study,

we have demonstrated that cell surface ATP synthase is involved in shear stress-induced ATP release. Immunofluorescence staining of human pulmonary arterial ECs (HPAECs) showed that cell surface ATP synthase is distributed in lipid rafts and co-localized with caveolin-1, a marker protein of caveolae. Immunoprecipitation indicated that cell surface ATP synthase and caveolin-1 are physically associated. Measurement of the extracellular metabolism of [^{3}H]ADP confirmed that cell surface ATP synthase is active in ATP generation. When exposed to shear stress, HPAECs released ATP in a dose-dependent manner, and the ATP release was markedly suppressed by the membrane-impermeable ATP synthase inhibitors angiostatin and piceatannol and by an anti-ATP synthase antibody. Depletion of plasma membrane cholesterol with methyl-β-cyclodextrin (MβCD) disrupted lipid rafts and abolished co-localization of ATP synthase with caveolin-1, which resulted in a marked reduction in shear stress-induced ATP release. Pretreatment of the cells with cholesterol prevented these effects of MβCD. Downregulation of caveolin-1 expression by transfection of caveolin-1 siRNA also markedly suppressed ATP-releasing responses to shear stress. Neither MβCD, MβCD plus cholesterol, nor caveolin-1 siRNA had any effect on the amount of cell surface ATP synthase. These results suggest that the localization and targeting of ATP synthase to caveolae/lipid rafts is critical for shear stress-induced ATP release by HPAECs.

3.962 Lipid Raft–Specific Knockdown of Src Family Kinase Activity Inhibits Cell Adhesion and Cell Cycle Progression of Breast Cancer Cells
Hitosugi, T., Sato, M., Sasaki, K and Umezawa, Y.
Cancer Res., ***(67/17)***, *8139-8148 (2007)*

Src family kinase (SFK) is known to control various cell functions, but the significance of the location of its activation was largely unknown. We herein revealed that SFK activation occurs in lipid rafts. Based on this finding, we have developed a lipid raft–targeted SFK inhibitory fusion protein (LRT-SIFP) that inhibits the SFK activity in lipid rafts. LRT-SIFP has a peptide inhibitor of SFK and a lipid raft–targeting sequence in which two cysteine residues are palmitoylated for clustering in lipid rafts. LRT-SIFP was found to inhibit cell adhesion and cell cycle progression of human breast cancer cell lines MCF-7 and MDA-MB231. On the other hand, the cell functions of MCF-7 cells were found to be not affected with a previously developed peptide inhibitor of SFK that lacks the lipid raft–targeting sequence. In addition, when we replaced the targeting sequence of LRT-SIFP with the consensus sequence for geranylgeranylation to make LRT-SIFP unable to cluster in lipid rafts, this mutated LRT-SIFP did not show any effect on the above cell functions of MCF-7 cells. Furthermore, in contrast to the breast cancer cell lines, LRT-SIFP did not show any inhibitory effect on cell adhesion and cell cycle progression of human normal cell line HEK293. The present lipid raft–specific knockdown of SFK activity would potentially be useful for selective cancer therapy to prevent tumorigenesis and metastasis of breast cancer cells.

3.963 Intracellular Trafficking of Pseudomonas ExoS, a Type III Cytotoxin
Deng, Q., Zhang, Y. and Barbier, J.T.
Traffic, ***8***, *1331-1345 (2007)*

Pseudomonas aeruginosa ExoS is a bifunctional type III cytotoxin that disrupts Ras- and Rho-signaling pathways in mammalian cells. A hydrophobic region (residues 51–77, termed the membrane localization domain) targets ExoS to the plasma membrane (PM) and late endosomes of host cells. In the current study, metabolic inhibitors and dominant-negative proteins that disrupt known vesicle-trafficking pathways were used to define the intracellular trafficking of ExoS. Release of ExoS from PM was independent of dynamin and ADP ribosylation factor 6 but inhibited by methyl-β-cyclodextrin, a cholesterol-depleting reagent, and perinuclear localization of ExoS was disrupted by nocodazole. p50 dynamitin, a dynein inhibitor partially disrupted perinuclear localization of ExoS. Methyl-β-cyclodextrin and nocodazole inhibited the ability of type-III-delivered ExoS to ADP-ribosylated Golgi/endoplasmic reticulum-resident Ras. Methyl-β-cyclodextrin also relocated ExoS from the perinuclear region to the PM, indicating that ExoS can cycle through anterograde as well as through retrograde trafficking pathways. These findings show that ExoS endocytosis is cholesterol dependent, and it utilizes host microtubules, for intracellular trafficking. Understanding how type III cytotoxins enter and traffic within mammalian cells may identify new targets for therapeutic intervention of gram-negative bacterial pathogens.

3.964 Biochemical consequences of the NOS3 Glu298Asp variation in human endothelium: altered caveolar localization and impaired response to shear
Joshi, M.S., Mineo, C., Shaul, P.W. and Bauer, J.A.
FASEB J., ***21***, *2655-2663 (2007)*

Human endothelial nitric oxide synthase (NOS3) gene polymorphism at Exon 7 (Glu298Asp) has been linked to vascular endothelial dysfunction, but the mechanisms are not defined. Shear is a key modulator of NOS3 function *in vivo* and association with caveolae is important for the control of NOS3 protein activity. Here we tested the hypothesis that altered enrichment of NOS3 in the caveolar membrane defines Glu298Asp genotype-specific responses and NOS3 activity. Basal caveolar membrane enrichment was carried out to quantitate the NOS3 enrichment in caveolae. Cells were subjected to shear and NOS3 protein levels, phosphorylation, enzyme function were investigated. Variant genotypes had lower NOx production pre- and post-shear, but no genotype-dependent alterations in pNOS3 were observed. Asp variants had significantly lower NOS3 enrichment in the caveolar membrane fraction. Further, immunoprecipitation studies demonstrated that Asp variants had substantially less NOS3/Cav-1 association (~40%) during static conditions. Furthermore, acute shear causes impaired NOS3/Cav-1 dissociation in Asp variants. The results from immunoprecipitation studies were in complete agreement with caveolar membrane preparation findings. Collectively, these data demonstrate functional consequences of the Glu298Asp NOS3 variation and further define disruption of NOS3 caveolar localization and shear-induced mobilization as the primary mechanism responsible for these differences.—Joshi, M. S., Mineo, C., Shaul, P. W., Bauer, J. A. Biochemical consequences of the NOS3 Glu298Asp variation in human endothelium: altered caveolar localization and impaired response to shear.

3.965 Peroxisomes Contain a Specific Phytanoyl-CoA/Pristanoyl-CoA Thioesterase Acting as a Novel Auxiliary Enzyme in α- and β-Oxidation of Methyl-branched Fatty Acids in Mouse
Westin, M.A., Hunt, M.C. and Elexson, S.E.H.
J. Biol. Chem., ***282(37)***, *26707-26716 (2007)*

Phytanic acid and pristanic acid are derived from phytol, which enter the body via the diet. Phytanic acid contains a methyl group in position three and, therefore, cannot undergo β-oxidation directly but instead must first undergo α-oxidation to pristanic acid, which then enters β-oxidation. Both these pathways occur in peroxisomes, and in this study we have identified a novel peroxisomal acyl-CoA thioesterase named ACOT6, which we show is specifically involved in phytanic acid and pristanic acid metabolism. Sequence analysis of ACOT6 revealed a putative peroxisomal targeting signal at the C-terminal end, and cellular localization experiments verified it as a peroxisomal enzyme. Subcellular fractionation experiments showed that peroxisomes contain by far the highest phytanoyl-CoA/pristanoyl-CoA thioesterase activity in the cell, which could be almost completely immunoprecipitated using an ACOT6 antibody. *Acot6* mRNA was mainly expressed in white adipose tissue and was co-expressed in tissues with *Acox3* (the pristanoyl-CoA oxidase). Furthermore, *Acot6* was identified as a target gene of the peroxisome proliferator-activated receptor α(PPARα) and is up-regulated in mouse liver in a PPARα-dependent manner.

3.966 Nuclear Import and Export of Venezuelan Equine Encephalitis Virus Nonstructural Protein 2
Montgomery, S.A. and Johnston, R.E.
J. Virol., ***81(19)***, *10268-10279 (2007)*

Many RNA viruses, which replicate predominantly in the cytoplasm, have nuclear components that contribute to their life cycle or pathogenesis. We investigated the intracellular localization of the multifunctional nonstructural protein 2 (nsP2) in mammalian cells infected with Venezuelan equine encephalitis virus (VEE), an important, naturally emerging zoonotic alphavirus. VEE nsP2 localizes to both the cytoplasm and the nucleus of mammalian cells in the context of infection and also when expressed alone. Through the analysis of a series of enhanced green fluorescent protein fusions, a segment of nsP2 that completely localizes to the nucleus of mammalian cells was identified. Within this region, mutation of the putative nuclear localization signal (NLS) PGKMV diminished, but did not obliterate, the ability of the protein to localize to the nucleus, suggesting that this sequence contributes to the nuclear localization of VEE nsP2. Furthermore, VEE nsP2 specifically interacted with the nuclear import protein karyopherin-α1 but not with karyopherin-α2, -3, or -4, suggesting that karyopherin-α1 transports nsP2 to the nucleus during infection. Additionally, a novel nuclear export signal (NES) was identified, which included residues L526 and L528 of VEE nsP2. Leptomycin B treatment resulted in nuclear accumulation of nsP2, demonstrating that nuclear export of nsP2 is mediated via the CRM1 nuclear export pathway. Disruption of either the NLS or the NES in nsP2 compromised essential viral functions. Taken together, these results establish the bidirectional transport of nsP2 across the nuclear membrane, suggesting that a critical function of nsP2 during infection involves its shuttling between the cytoplasm and the nucleus.

3.967 Association of the Astrovirus Structural Protein VP90 with Membranes Plays a Role in Virus Morphogenesis

Mendez, E., Aguirre-Crespo, G., Zavala, G. and Arias, C.F.
J. Virol., ***81(19)***, *10649-10658 (2007)*

VP90, the capsid polyprotein precursor of human astrovirus Yuc8, is assembled into viral particles, and its processing at the carboxy terminus by cellular caspases, to yield VP70, has been correlated with the cell release of the virus. Here, we characterized the effect of the VP90-VP70 processing on the properties of these proteins, as well as on their intracellular distribution. VP90 was found in membrane-enriched fractions (^{m}VP90), as well as in fractions enriched in cytosolic proteins (^{c}VP90), while VP70 was found exclusively in the latter fractions. Upon trypsin activation, infectivity was detected in all VP90-containing fractions, confirming that both ^{m}VP90 and ^{c}VP90 are able to assemble into particles; however, the two forms of VP90 showed differential sensitivities to trypsin, especially at their carboxy termini, which in the case of ^{m}VP90 was shown to remain membrane associated after protease digestion. Structural protein oligomers were detected in purified VP70-containing viruses, as well as in membrane-enriched fractions, but they were less evident in cytosolic fractions. Ultrastructural studies of infected cells revealed different types of viral particles, some of which appeared to be associated with membranes. By immunoelectron microscopy, structural proteins were shown to form virus particles in clusters and to associate with the edges of vesicles induced during infection, which also appear to contain subviral particles inside. Nonstructural proteins and viral RNA colocalized with ^{m}VP90, but not with ^{c}VP90, suggesting that ^{m}VP90 might represent the form of the protein that is initially assembled into particles, at the sites where the virus genome is being replicated.

3.968 Intracellular HIV-1 Gag localization is impaired by mutations in the nucleocapsid zinc fingers

Grigorov, B. et al
Retrovirology, ***4(54)***, *1-12 (2007)*

Background
The HIV-1 nucleocapsid protein (NC) is formed of two CCHC zinc fingers flanked by highly basic regions. HIV-1 NC plays key roles in virus structure and replication *via* its nucleic acid binding and chaperoning properties. In fact, NC controls proviral DNA synthesis by reverse transcriptase (RT), gRNA dimerization and packaging, and virion assembly.
Results
We previously reported a role for the first NC zinc finger in virion structure and replication [1]. To investigate the role of both NC zinc fingers in intracellular Gag trafficking, and in virion assembly, we generated series of NC zinc fingers mutations. Results show that all Zinc finger mutations have a negative impact on virion biogenesis and maturation and rendered defective the mutant viruses. The NC zinc finger mutations caused an intracellular accumulation of Gag, which was found either diffuse in the cytoplasm or at the plasma membrane but not associated with endosomal membranes as for wild type Gag. Evidences are also provided showing that the intracellular interactions between NC-mutated Gag and the gRNA were impaired.
Conclusion
These results show that Gag oligomerization mediated by gRNA-NC interactions is required for correct Gag trafficking, and assembly in HIV-1 producing cells and the release of infectious viruses.

3.969 Effects of a New Bioactive Lipid-Based Drug Carrier on Cultured Hepatic Stellate Cells and Liver Fibrosis in Bile Duct-Ligated Rats

Adrian, J.E. et al
J. Pharmacol. Exp. Ther., ***321(2)***, *536-543 (2007)*

In the fibrotic liver, hepatic stellate cells (HSC) produce large amounts of collagen and secrete variety of mediators that promote development of fibrosis in this organ. Therefore, these cells are considered an attractive target for antifibrotic therapies. We incorporated the bioactive lipid dilinoleoylphosphatidylcholine (DLPC) into the membrane of liposomes, and then we evaluated its effect on hepatic stellate cell activation and liver fibrosis. To target DLPC-liposomes to HSC, human serum albumin modified with mannose 6-phosphate (M6P-HSA) was coupled to the surface of these liposomes. In vitro, the effects of the carrier were determined in primary cultures of HSC, Kupffer cells, and liver endothelial cells using real-time reverse transcription-polymerase chain reaction. In vivo DLPC-liposomes were tested in bile duct-ligated rats. Targeted M6P-HSA-DLPC-liposomes and DLPC-liposomes significantly reduced gene expression levels for collagen 1α1, α-smooth muscle actin (α-SMA), and

transforming growth factor-β (TGF-β) in cultured HSC. In fibrotic livers, DLPC-liposomes decreased gene expression for TGF-β and collagen 1α1 as well as α-SMA and collagen protein expression. In contrast, M6P-HSA-DLPC-liposomes enhanced expression of profibrotic and proinflammatory genes in vivo. In cultured Kupffer and endothelial cells M6P-HSA liposomes influenced the expression of proinflammatory genes. Both types of liposomes increased hepatocyte glycogen content in fibrotic livers, indicating improved functionality of the hepatocytes. We conclude that DLPC-containing liposomes attenuate activation of cultured HSC. In fibrotic livers, M6P-HSA-mediated activation of Kupffer and endothelial cells probably counteracts this beneficial effect of DLPC-liposomes. Therefore, these bioactive drug carriers modulate the activity of all liver cells during liver fibrosis.

3.970 Characterization of the Intracellular Proteolytic Cleavage of Myocilin and Identification of Calpain II as a Myocilin-processing Protease
Sanchez-Sanchez, F., Martinez-Redondo, F., Aroca-Aguilar, J.D., Coca-Prados, M. and Escrobano, J.
J. Biol. Chem., ***282(38)***, *27810-27824 (2007)*

MYOC, a gene involved in different types of glaucoma, encodes myocilin, a secreted glycoprotein of unknown function, consisting of an N-terminal leucine-zipper-like domain, a central linker region, and a C-terminal olfactomedin-like domain. Recently, we have shown that myocilin undergoes an intracellular endoproteolytic processing. We show herein that the proteolytic cleavage in the linker region splits the two terminal domains. The C-terminal domain is secreted to the culture medium, whereas the N-terminal domain mainly remains intracellularly retained. In transiently transfected 293T cells, the cleavage was prevented by calpain inhibitors, such as calpeptin, calpain inhibitor IV, and calpastatin. Since calpains are calcium-activated proteases, we analyzed how changes in either intra- or extracellular calcium affected the cleavage of myocilin. Intracellular ionomycin-induced calcium uptake enhanced myocilin cleavage, whereas chelation of extracellular calcium by EGTA inhibited the proteolytic processing. Calpains I and II cleaved myocilin *in vitro*. However, in cells in culture, only RNA interference knockdown of calpain II reduced myocilin processing. Subcellular fractionation and digestion of the obtained fractions with proteinase K showed that full-length myocilin resides in the lumen of the endoplasmic reticulum together with a subpopulation of calpain II. These data revealed that calpain II is responsible for the intracellular processing of myocilin in the lumen of the endoplasmic reticulum. We propose that this cleavage might regulate extracellular interactions of myocilin, contributing to the control of intraocular pressure.

3.971 HtrA2 Regulates β-Amyloid Precursor Protein (APP) Metabolism through Endoplasmic Reticulum-associated Degradation
Huttunen, H.J. et al
J. Biol. Chem., ***282(38)***, *28285-28295 (2007)*

Alzheimer disease-associated β-amyloid peptide is generated from its precursor protein APP. By using the yeast two-hybrid assay, here we identified HtrA2/Omi, a stress-responsive chaperone-protease as a protein binding to the N-terminal cysteinerich region of APP. HtrA2 coimmunoprecipitates exclusively with immature APP from cell lysates as well as mouse brain extracts and degrades APP *in vitro*. A subpopulation of HtrA2 localizes to the cytosolic side of the endoplasmic reticulum (ER) membrane where it contributes to ER-associated degradation of APP together with the proteasome. Inhibition of the proteasome results in accumulation of retrotranslocated forms of APP and increased association of APP with HtrA2 and Derlin-1 in microsomal membranes. In cells lacking HtrA2, APP holoprotein is stabilized and accumulates in the early secretory pathway correlating with elevated levels of APP C-terminal fragments and increased Aβ secretion. Inhibition of ER-associated degradation (either HtrA2 or proteasome) promotes binding of APP to the COPII protein Sec23 suggesting enhanced trafficking of APP out of the ER. Based on these results we suggest a novel function for HtrA2 as a regulator of APP metabolism through ER-associated degradation.

3.972 Anionic Lipid Interaction Induces Prion Protein Conformational Change
Wang, F., Wang, X. and Ma, J.
FASEB J., ***21***, *781.3 (2007)*

The conversion of the prion protein (PrP) to the pathogenic PrP^{Sc} conformation plays a central role in prion disease. However, the precise mechanism underlying this process remains unclear. Here, we report the conformational conversion of PrP upon interaction with anionic lipids. After the discontinuous **iodixanol**

density gradient centrifugation, we found strong binding between PrP and negatively charged phospholipids, involving both electrostatic and hydrophobic interactions. Under physiologically relevant conditions, interactions with lipid were sufficient to convert full-length, α-helices rich recombinant mouse PrP to a conformation similar to PrP^{Sc}, with increased ß sheet content and a PrP^{Sc}-like proteinase K (PK)-resistant pattern. Conversion is greatly influenced by lipid headgroup structures and lipid vesicle compositions. When lipid vesicles are disrupted by detergent, aggregation is necessary to maintain the PK resistant conformation. Our results imply that the strong lipid-PrP interaction is sufficient to overcome the energy barrier between the two conformational states and support the notion that lipid membrane may play a role in PrP conformational change.

3.973 SVIP interacts with Derlin1 and regulates ER-associated degradation
Ballar, P. et al
FASEB J., ***21***, *808.3 (2007)*

Production of misfolded proteins in the endoplasmic reticulum (ER) underlies pathogenesis of many diseases. Cells utilize a process, called ER-associated degradation (ERAD) to eliminate misfolded ER proteins, thereby protecting against the toxicity of the defective proteins. However, the molecular mechanisms underlying the process of ERAD remain to be fully understood. Here, we report that the SVIP is specifically and highly expressed in mouse brain as revealed by mouse multi-tissue blotting. By **Opti-Prep** density gradient fractionation, we demonstrated that SVIP is co-fractionated with ERAD machineries, including Derlin1, Hrd1, and gp78. Co-immunoprecipitation shows that SVIP strongly interacts with Derlin1 and p97/VCP, but weakly with Hrd1 and gp78. SVIP is anchored to the ER membrane probably via myristoylation, since mutation of the putative myristoylation site impaired its ER localization. Further, we found that SVIP is upregulated by tunicamycin-induced ER stress both at mRNA and protein levels. Functionally, SVIP enhances the loading of polyubiquitinated proteins to p97/VCP, suggesting that SVIP may play a role in coupling ubiquitination with retrotranslocation of misfolded proteins during ERAD. Consistently, silencing of SVIP expression by RNA interference stabilizes ERAD substrate tyrosinase(C89R). These results suggest that SVIP may facilitate the coupling of ubiquitination with retrotranslocation during ERAD and might play a protective role in brain.

3.974 CFTR Inhibitory Factor (CIF) reduces the plasma membrane expression of CFTR by altering intracellular trafficking of CFTR to the lysosomal pathway
Bomberger, J.M. et al
FASEB J., ***21***, *944.4 (2007)*

The ΔF508-CFTR mutation, the most common gene mutation in cystic fibrosis (CF), results in diminished plasma membrane expression of CFTR, leading to loss of functional CFTR and altered mucociliary clearance. This impairment promotes chronic infection of CF patients by *P. aeruginosa*. Previously we reported that a secreted factor from *P. aeruginosa* (CIF) reduces CFTR-mediated chloride secretion and the plasma membrane expression of CFTR by decreasing endocytic recycling. The aim of the current study was to investigate the mechanism by which CIF reduces the endocytic recycling of CFTR. CIF applied to the apical side of polarized human airway epithelial cells reduced CFTR in the plasma membrane, followed by a subsequent increase in CFTR labeling in the endosomes, then lysosomes, as determined by **Optiprep** subcellular compartment fractionation experiments. Co-immunoprecipitation studies revealed that CIF decreased the association of CFTR with Rab11a, concurrent with an increase in association of CFTR with Rab4a and Rab7. Lysosomal inhibitors blocked the degradation of CFTR by CIF, whereas proteosomal inhibitors had no effect. These studies demonstrate that CIF induces a redistribution of CFTR trafficking from the endocytic recycling pathway to the degradative pathway. In addition, this data suggests that chronic infection of *P. aeruginosa* in the CF lung may impact the efficacy of therapeutics developed to increase plasma membrane expression of the ΔF508-CFTR.

3.975 Cellular spelunking: exploring adipocyte caveolae
Pilch, P.F. et al
J. Lipid Res., ***48***, *2103-2111 (2007)*

It has been known for decades that the adipocyte cell surface is particularly rich in small invaginations we now know to be caveolae. These structures are common to many cell types but are not ubiquitous. They have generated considerable curiosity, as manifested by the numerous publications on the topic that describe various, sometimes contradictory, caveolae functions. Here, we review the field from an "adipocentric" point of view and suggest that caveolae may have a function of particular use for the fat cell,

namely the modulation of fatty acid flux across the plasma membrane. Other functions for adipocyte caveolae that have been postulated include participation in signal transduction and membrane trafficking pathways, and it will require further experimental scrutiny to resolve controversies surrounding these possible activities.

3.976 Lifeguard/neuronal membrane protein 35 regulates Fas ligand-mediated apoptosis in neurons via microdomain recruitment
Fernandez, M. et al
J. Neurochem., ***103***, *190-203 (2007)*

Fas ligand (FasL)-receptor system plays an essential role in regulating cell death in the developing nervous system, and it has been implicated in neurodegenerative and inflammatory responses in the CNS. Lifeguard (LFG) is a protein highly expressed in the hippocampus and the cerebellum, and it shows a particularly interesting regulation by being up-regulated during postnatal development and in the adult. We show that over-expression of LFG protected cortical neurons from FasL-induced apoptosis and decreased caspase-activation. Reduction of endogenous LFG expression by small interfering RNA sensitized cerebellar granular neurons to FasL-induced cell death and caspase-8 activation, and also increased sensitivity of cortical neurons. In differentiated cerebellar granular neurons, protection from FasL-induced cell death could be attributed exclusively to LFG and appears to be independent of FLICE inhibitor protein. Thus, LFG is an endogenous inhibitor of FasL-mediated neuronal death and it mediates the FasL resistance of CNS differentiated neurons. Finally, we also demonstrate that LFG is detected in lipid rafts microdomains, where it may interact with Fas receptor and regulate FasL-activated signaling pathways.

3.977 Mannheimia haemolytica Leukotoxin Binds to Lipid Rafts in Bovine Lymphoblastoid Cells and Is Internalized in a Dynamin-2- and Clathrin-Dependent Manner
Atapatta, D.N. and Czuprynski, C.J.
Infect. Immun., ***75(10)***, *4719-4727 (2007)*

Mannheimia haemolytica is the principal bacterial pathogen of the bovine respiratory disease complex. Its most important virulence factor is a leukotoxin (LKT), which is a member of the RTX family of exotoxins produced by many gram-negative bacteria. Previous studies demonstrated that LKT binds to the ß$_2$-integrin LFA-1 (CD11a/CD18) on bovine leukocytes, resulting in cell death. In this study, we demonstrated that depletion of lipid rafts significantly decreases LKT-induced bovine lymphoblastoid cell (BL-3) death. After binding to BL-3 cells, some of the LKT relocated to lipid rafts in an LFA-1-independent manner. We hypothesized that after binding to LFA-1 on BL-3 cells, LKT moves to lipid rafts and clathrin-coated pits via a dynamic process that results in LKT internalization and cytotoxicity. Knocking down dynamin-2 by small interfering RNA reduced both LKT internalization and cytotoxicity. Similarly, expression of dominant negative Eps15 protein expression, which is required for clathrin coat formation, reduced LKT internalization and LKT-mediated cytotoxicity to BL-3 cells. Finally, we demonstrated that inhibiting actin polymerization reduced both LKT internalization and LKT-mediated cytotoxicity. These results suggest that both lipid rafts and clathrin-mediated mechanisms are important for LKT internalization and cytotoxicity in BL-3 cells and illustrate the complex nature of LKT internalization by the cytoskeletal network.

3.978 Phosphoinositide 3-Kinase-independent Non-genomic Signals Transit from the Androgen Receptor to Akt1 in Membrane Raft Microdomains
Cinar, B., Mukhopadhyay, N.K., Meng, G. and Freeman, M.R.
J. Biol. Chem., ***282(40)***, *29584-29593 (2007)*

The serine-threonine kinase, Akt1/protein kinase Bα is an important mediator of growth, survival, and metabolic signaling. Recent studies have implicated cholesterol-rich, lipid raft microdomains in survival signals mediated by Akt1. Here we address the role of lipid raft membranes as a potential site of intersection of androgenic and Akt1 signaling. A subpopulation of androgen receptor (AR) was found to localize to a lipid raft subcellular compartment in LNCaP prostate cancer cells. Endogenous AR interacted with endogenous Akt1 preferentially in lipid raft fractions and androgen substantially enhanced the interaction between the two proteins. The association of AR with Akt1 was inhibited by the anti-androgen, bicalutamide, but was not affected by inhibition of phosphoinositide 3-kinase (PI3K). Androgen promoted endogenous Akt1 activity in lipid raft fractions, in a PI3K-independent manner, within 10 min of treatment. Fusion of a lipid raft targeting sequence to AR enhanced localization of the receptor to rafts, and stimulated Akt1 activity in response to androgen, while reducing the cells' dependence on constitutive signaling

through PI3K for cell survival. These findings suggest that signals channeled through AR and Akt1 intersect by a mechanism involving formation within lipid raft membranes of an androgen-responsive, extranuclear AR/Akt1 complex. Our results indicate that cholesterol-rich membrane microdomains play a role in transmitting non-genomic signals involving androgen and the Akt pathway in prostate cancer cells.

3.979 Ablation of a small transmembrane protein of Trypanosoma brucei (TbVTC1) involved in the synthesis of polyphosphate alters acidocalcisome biogenesis and function, and leads to a cytokinesis defect

Fang, J., Rohloff, P., Miranda, K. And Docampo, R.
Biochem. J., ***407***, *161-170 (2007)*

Inorganic poly P (polyphosphate) is an abundant component of acidocalcisomes of *Trypanosoma brucei*. In the present study we report the presence of a protein homologous with the yeast Vtc1p (vacuolar transporter chaperone 1) in *T. brucei* that is essential for poly P synthesis, acidocalcisome biogenesis and cytokinesis. Localization studies in a cell line expressing a TbVTC1 fused to GFP (green fluorescent protein) revealed its co-localization with the V-H^+-PPase (vacuolar H^+-pyrophosphatase), a marker for acidocalcisomes. Western blot analysis of acidocalcisome fractions and immunogold electron microscopy using polyclonal antibodies against a fragment of TbVTC1 confirmed the acidocalcisome localization. Ablation of *TbVTC1* expression by RNA interference caused an abnormal morphology of acidocalcisomes, indicating that their biogenesis was disturbed, with a decreased pyrophosphate-driven H^+ uptake and Ca^{2+} content, a significant decrease in the amount of poly P and a deficient response to hyposmotic stress. Ablation of *TbVTC1* expression for longer periods produced marked gross morphological alterations compatible with a defect in cytokinesis, followed by cell death. Overexpression of the *TbVTC1* gene caused mild alterations in growth rate, but had no perceptible effect on acidocalcisome morphology. We propose that the PP_i-driven H^+ pumping deficiency induced by ablation of *TbVTC1* leads to alterations in the protonmotive force of acidocalcisomes, which results in deficient fusion or budding of the organelles, decreased H^+ and Ca^{2+} content, and decreased synthesis of poly P. A decrease in the poly P content would lead to osmotic sensitivity and defects in cytokinesis.

3.980 Peroxisomal-mitochondrial oxidation in a rodent model of obesity-associated insulin resistance

Noland, R.C. et al
Am. J. Physiol. Endocrinol. Metab., ***293***, *E986-E1001 (2007)*

Peroxisomal oxidation yields metabolites that are more efficiently utilized by mitochondria. This is of potential clinical importance because reduced fatty acid oxidation is suspected to promote excess lipid accumulation in obesity-associated insulin resistance. Our purpose was to assess peroxisomal contributions to mitochondrial oxidation in mixed gastrocnemius (MG), liver, and left ventricle (LV) homogenates from lean and fatty (*fa/fa*) Zucker rats. Results indicate that complete mitochondrial oxidation (CO_2 production) using various lipid substrates was increased approximately twofold in MG, unaltered in LV, and diminished ~50% in liver of *fa/fa* rats. In isolated mitochondria, malonyl-CoA inhibited CO_2 production from palmitate 78%, whereas adding isolated peroxisomes reduced inhibition to 21%. These data demonstrate that peroxisomal products may enter mitochondria independently of CPT I, thus providing a route to maintain lipid disposal under conditions where malonyl-CoA levels are elevated, such as in insulin-resistant tissues. Peroxisomal metabolism of lignoceric acid in *fa/fa* rats was elevated in both liver and MG (LV unaltered), but peroxisomal product distribution varied. A threefold elevation in incomplete oxidation was solely responsible for increased hepatic peroxisomal oxidation (CO_2 unaltered). Alternatively, only CO_2 was detected in MG, indicating that peroxisomal products were exclusively partitioned to mitochondria for complete lipid disposal. These data suggest tissue-specific destinations for peroxisome-derived products and emphasize a potential role for peroxisomes in skeletal muscle lipid metabolism in the obese, insulin-resistant state.

3.981 Anti–ß2-microglobulin monoclonal antibodies induce apoptosis in myeloma cells by recruiting MHC class I to and excluding growth and survival cytokine receptors from lipid rafts

Yang, J. et al
Blood, ***110(8)****, 3028-3035 (2007)*

We recently showed that monoclonal antibodies (mAbs) against $ß_2$-microglobulin ($ß_2$M) have a remarkably strong apoptotic effect on myeloma cells. The mAbs induced apoptosis by recruiting major histocompatibility complex (MHC) class I to lipid rafts, activated c-Jun N-terminal kinase (JNK), and inhibited phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal–regulated kinase (ERK)

pathways. Growth and survival cytokines such as interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-I), which could protect myeloma cells from dexamethasone-induced apoptosis, did not affect mAb-mediated cell death. This study was undertaken to elucidate the mechanisms underlying anti-ß$_2$M mAb–induced PI3K/Akt and ERK inhibition and the inability of IL-6 and IGF-I to protect myeloma cells from mAb-induced apoptosis. We focused on lipid rafts and confirmed that these membrane microdomains are required for IL-6 and IGF-I signaling. By recruiting MHC class I into lipid rafts, anti-ß$_2$M mAbs excluded IL-6 and IGF-I receptors and their substrates from the rafts. The mAbs not only redistributed the receptors in cell membrane, but also abrogated IL-6– or IGF-I–mediated Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3), PI3K/Akt, and Ras/Raf/ERK pathway signaling, which are otherwise constitutively activated in myeloma cells. Thus, this study further defines the tumoricidal mechanism of the mAbs and provides strong evidence to support the potential of these mAbs as therapeutic agents for myeloma.

3.982 **Protein kinase C-ε coimmunoprecipitates with cytochrome oxidase subunit IV and is associated with improved cytochrome-c oxidase activity and cardioprotection**
Guo, D. et al
Am. J. Physiol. Heart Circ. Physiol., ***293***, *H2219-H2230 (2007)*

We have utilized an in situ rat coronary ligation model to establish a PKC-ε cytochrome oxidase subunit IV (COIV) coimmunoprecipitation in myocardium exposed to ischemic preconditioning (PC). Ischemia-reperfusion (I/R) damage and PC protection were confirmed using tetrazolium-based staining methods and serum levels of cardiac troponin I. Homogenates prepared from the regions at risk (RAR) and not at risk (RNAR) for I/R injury were fractionated into cell-soluble (S), 600 *g* low-speed centrifugation (L), Percoll/**Optiprep** density gradient-purified mitochondrial (M), and 100,000 *g* particulate (P) fractions. COIV immunoreactivity and cytochrome-*c* oxidase activity measurements estimated the percentages of cellular mitochondria in S, L, M, and P fractions to be 0, 55, 29, and 16%, respectively. We observed 18, 3, and 3% of PKC-δ, -ε, and -ζ isozymes in the M fraction under basal conditions. Following PC, we observed a 61% increase in PKC-ε levels in the RAR M fraction compared with the RNAR M fraction. In RAR mitochondria, we also observed a 2.8-fold increase in PKC-ε serine 729 phosphoimmunoreactivity (autophosphorylation), indicating the presence of activated PKC-ε in mitochondria following PC. PC administered before prolonged I/R induced a 1.9-fold increase in the coimmunoprecipitation of COIV, with anti-PKC-ε antisera and a twofold enhancement of cytochrome-*c* oxidase activity. Our results suggest that PKC-ε may interact with COIV as a component of the cardioprotection in PC. Induction of this interaction may provide a novel therapeutic target for protecting the heart from I/R damage.

3.983 **HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis**
Lin, L et al
Am. J. Physiol. Heart Circ. Physiol., ***293***, *H2238-H2247 (2007)*

Heat shock protein (HSP) 60 is a mitochondrial and cytosolic protein. Previously, we reported that HSP60 doubled in end-stage heart failure, even though levels of the protective HSP72 were unchanged. Furthermore, we observed that acute injury in adult cardiac myocytes resulted in movement of HSP60 to the plasma membrane. We hypothesized that the inflammatory state of heart failure would cause translocation of HSP60 to the plasma membrane and that this would provide a pathway for cardiac injury. Two models were used to test this hypothesis: *1*) a rat model of heart failure and *2*) human explanted failing hearts. We found that HSP60 localized to the plasma membrane and was also found in the plasma early in heart failure. Plasma membrane HSP60 localized to lipid rafts and was detectable on the cell surface with the use of both flow cytometry and confocal microscopy. Localization of HSP60 to the cell surface correlated with increased apoptosis. In heart failure, HSP60 is in the plasma membrane fraction, on the cell surface, and in the plasma. Membrane HSP60 correlated with increased apoptosis. Release of HSP60 may activate the innate immune system, promoting a proinflammatory state, including an increase in TNF-α. Thus abnormal trafficking of HSP60 to the cell surface may be an early trigger for myocyte loss and the progression of heart failure.

3.984 **Metabolism and short-term metabolic effects of conjugated linoleic acids in rat hepatocytes**
Priore, P., Giudetti, A.M., Natali, F., Gnoni, G.V. and Geelen, M.J.H.
Biochim. Biophys. Acta, ***1771(10)***, *1299-1307 (2007)*

Metabolic fate and short-term effects of a 1:1 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12-conjugated linoleic acids (CLA), compared to linoleic acid (LA), on lipid metabolism was investigated in rat liver. In

isolated mitochondria CLA-CoA were poorer substrates than LA-CoA for carnitine palmitoyltransferase-I (CPT-I) activity. However, in digitonin-permeabilized hepatocytes, where interactions among different metabolic pathways can be simultaneously investigated, CLA induced a remarkable stimulatory effect on CPT-I activity. This stimulation can be ascribed to a reduced malonyl-CoA level in turn due to inhibition of acetyl-CoA carboxylase (ACC) activity. The ACC/malonyl-CoA/CPT-I system can therefore represent a coordinate control by which CLA may exert effects on the partitioning of fatty acids between esterification and oxidation. Moreover, the rate of oxidation to CO_2 and ketone bodies was significantly higher from CLA; peroxisomes rather than mitochondria were responsible for this difference. Interestingly, peroxisomal acyl-CoA oxidase (AOX) activity strongly increased by CLA-CoA compared to LA-CoA. CLA, metabolized by hepatocytes at a higher rate than LA, were poorer substrates for cellular and VLDL-triacylglycerol (TAG) synthesis. Overall, our results suggest that increased fatty acid oxidation with consequent decreased fatty acid availability for TAG synthesis is a potential mechanism by which CLA reduce TAG level in rat liver.

3.985 Effect of hypoxia on the binding and subcellular distribution of iron regulatory proteins
Christova, T. and Templeton, D.M.
Mol. Cell. Biochem., ***301***, *21-32 (2007)*

Iron regulatory proteins 1 and 2 (IRP1, IRP2) are key determinants of uptake and storage of iron by the liver, and are responsive to oxidative stress and hypoxia potentially at the level of both protein concentration and mRNA-binding activity. We examined the effect of hypoxia (1% O_2) on IRP1 and IRP2 levels (Western blots) and mRNA-binding activity (gel shift assays) in human hepatoma HepG2 cells, and compared them with HEK 293 cells, a renal cell line known to respond to hypoxia. Total IRP binding to an iron responsive element (IRE) mRNA probe was increased several fold by hypoxia in HEK 293 cells, maximally at 4–8 h. An earlier and more modest increase (1.5- to 2-fold, peaking at 2 h and then declining) was seen in HepG2 cells. In both cell lines, IRP1 made a greater contribution to IRE-binding activity than IRP2. IRP1 protein levels were increased slightly by hypoxia in HEK 293 but not in HepG2 cells. IRP1 was distributed between cytosolic and membrane-bound fractions, and in both cells hypoxia increased both the amount and IRE-binding activity of the membrane-associated IRP1 fraction. Further density gradient fractionation of HepG2 membranes revealed that hypoxia caused an increase in total membrane IRP1, with a shift in the membrane-bound fraction from Golgi to an endoplasmic reticulum (ER)-enriched fraction. Translocation of IRP to the ER has previously been shown to stabilize transferrin receptor mRNA, thus increasing iron availability to the cell. Iron depletion with deferoxamine also caused an increase in ER-associated IRP1. Phorbol ester caused serine phosphorylation of IRP1 and increased its association with the ER. The calcium ionophore ionomycin likewise increased ER-associated IRP1, without affecting total IRE-binding activity. We conclude that IRP1 is translocated to the ER by multiple signals in HepG2 cells, including hypoxia, thereby facilitating its role in regulation of hepatic gene expression.

3.986 Release of thioredoxin from Saccharomyces cerevisiae with environmental stimuli: solubilization of thioredoxin with ethanol
Takeuchi, Y. et al
Appl. Microbiol. Biotechnol., ***75***, *1393-1399 (2007)*

Thioredoxin is crucial for the maintenance of the redox status of cells of all types. Mammalian thioredoxin is secreted from various types of cells, although the mechanism underlying has not yet been clarified. Previously, we demonstrated that thioredoxin was released from *Saccharomyces cerevisiae* after treatment with ethanol. In this paper, we show that as well as ethanol, low-pH shock and hypoosmotic shock release thioredoxin. Low-molecular-weight proteins in yeast cells were preferentially released by treatment with ethanol and low-pH shock. A cell wall integrity pathway seems partially involved in the hypoosmotic shock-induced release of thioredoxin. Considerable amounts of thioredoxin were present in the insoluble fractions of the cells, a portion of which was associated with lipid microdomains that are resistant to nonionic detergent at 4°C. The intracellular localization of thioredoxin may influence the efficiency of its release from yeast cells with ethanol.

3.987 Flagellar membrane trafficking in kinetoplastids
Fridberg, A., Buchanan, K.T. and Engman, D.M.
Parasitol. Res., ***100***, *205-212 (2007)*

This review was presented at the International Symposium on Vesicle Trafficking in Parasitic Protozoa at Caxambu, Brasil, in November, 2005.

3.988 Influence of lipid rafts on CD1d presentation by dendritic cells
Peng, W. et al
Mol. Membrane Biol., ***24(5-6)***, *475-484 (2007)*

Our main objective was to analyze the role of lipid rafts in the activation of Vα-14$^-$ and Vα-14$^+$ T hybridomas by dendritic cells. We showed that activation of Vα-14$^+$ hybridomas by dendritic cells or other CD1d-expressing cells was altered by disruption of lipid rafts with the cholesterol chelator MβCD. However, CD1d presentation to autoreactive Vα-14$^-$ anti-CD1d hybridomas which do not require the endocytic pathway was not altered. Using partitioning of membrane fractions with Brij98 at 37°C, we confirmed that CD1d was enriched in subcellular fractions corresponding to lipid rafts and we describe that α-GalCer enhanced CD1d amount in the low density detergent insoluble fraction. We conclude that the membrane environment of CD1d can influence antigen presentation mainly when the endocytic pathway is required. Flow cytometry analysis can provide additional information on lipid rafts in plasma membranes and allows a dynamics follow-up of lipid rafts partitioning. Using this method, we showed that CD1d plasma membrane expression was sensitive to low concentrations of detergent. This may suggest either that CD1d is associated with lipid rafts mainly in intracellular membranes or that its association with the lipid rafts in the plasma membrane is weak.

3.989 Lipid Raft Proteomics: More than Just Detergent-Resistant Membranes
Foster, L.J. and Chan, Q.W.T.
Subcellular Proteomics, ***43***, *35-47 (2007)*

No abstract available

3.990 Plasma Membrane Proteomics
Alexandersson, E., Gustavsson, N., Bernfur, K., Kjellbom, P. and Larsson, C.
Plant Proteomics, Springer Berlin/Heidelberg, 189-206 (2007)

Proteins residing in the plasma membrane have key functions in transport, signal transduction, vesicle trafficking and many other important processes. To better understand these processes it is necessary to reveal the identity of plasma membrane proteins and to monitor modifications and regulation of their expression. This chapter is an overview of the methods used in plant plasma membrane proteomic studies and the results obtained so far. It focuses on studies using mass spectrometry for identification and includes aspects of plasma membrane fractionation, extraction and washing treatments, assessment of purity, separation methods for plasma membrane proteins and choice of techniques for protein cleavage. Finally, the results of plasma membrane proteomic studies are compared and problems with contaminating proteins are discussed.

3.991 CD44 Regulates Hepatocyte Growth Factor-mediated Vascular Integrity: ROLE OF c-Met, Tiam1/Rac1, DYNAMIN 2, AND CORTACTIN
Singleton, P.A. et al
J. Biol. Chem., ***282(42)***, *30643-30657 (2007)*

The preservation of vascular endothelial cell (EC) barrier integrity is critical to normal vessel homeostasis, with barrier dysfunction being a feature of inflammation, tumor angiogenesis, atherosclerosis, and acute lung injury. Therefore, agents that preserve or restore vascular integrity have important therapeutic implications. In this study, we explored the regulation of hepatocyte growth factor (HGF)-mediated enhancement of EC barrier function via CD44 isoforms. We observed that HGF promoted c-Met association with CD44v10 and recruitment of c-Met into caveolin-enriched microdomains (CEM) containing CD44s (standard form). Treatment of EC with CD44v10-blocking antibodies inhibited HGF-mediated c-Met phosphorylation and c-Met recruitment to CEM. Silencing CD44 expression (small interfering RNA) attenuated HGF-induced recruitment of c-Met, Tiam1 (a Rac1 exchange factor), cortactin (an actin cytoskeletal regulator), and dynamin 2 (a vesicular regulator) to CEM as well as HGF-induced

trans-EC electrical resistance. In addition, silencing Tiam1 or dynamin 2 reduced HGF-induced Rac1 activation, cortactin recruitment to CEM, and EC barrier regulation. We observed that both HGF- and high molecular weight hyaluronan (CD44 ligand)-mediated protection from lipopolysaccharide-induced pulmonary vascular hyperpermeability was significantly reduced in CD44 knock-out mice, thus validating these *in vitro* findings in an *in vivo* murine model of inflammatory lung injury. Taken together, these results suggest that CD44 is an important regulator of HGF/c-Met-mediated *in vitro* and *in vivo* barrier enhancement, a process with essential involvement of Tiam1, Rac1, dynamin 2, and cortactin.

3.992 **A reversible form of lysine acetylation in the ER and Golgi lumen controls the molecular stabilization of BACE1**
Costantini, C., Ko, M.H., Cabell, M., Jonas, M.C. and Puglielli, L.
Biochem. J., ***407***, *383-395 (2007)*

The lipid second messenger ceramide regulates the rate of β cleavage of the Alzheimer's disease APP (amyloid precursor protein) by affecting the molecular stability of the β secretase BACE1 (β-site APP cleaving enzyme 1). Such an event is stimulated in the brain by the normal process of aging, and is under the control of the general aging programme mediated by the insulin-like growth factor 1 receptor. In the present study we report that BACE1 is acetylated on seven lysine residues of the N-terminal portion of the nascent protein. This process involves lysine acetylation in the lumen of the ER (endoplasmic reticulum) and is followed by deacetylation in the lumen of the Golgi apparatus, once the protein is fully mature. We also show that specific enzymatic activities acetylate (in the ER) and deacetylate (in the Golgi apparatus) the lysine residues. This process requires carrier-mediated translocation of acetyl-CoA into the ER lumen and is stimulated by ceramide. Site-directed mutagenesis indicates that lysine acetylation is necessary for nascent BACE1 to leave the ER and move ahead in the secretory pathway, and for the molecular stabilization of the protein.

3.993 **A Functional Dynein–Microtubule Network Is Required for NGF Signaling Through the Rap1/MAPK Pathway**
Wu, C. et al
Traffic, ***8***, *1503-1520 (2007)*

Rap1 transduces nerve growth factor (NGF)/tyrosine receptor kinase A (TrkA) signaling in early endosomes, leading to sustained activation of the p44/p42 mitogen-activated protein kinases (MAPK1/2). However, the mechanisms by which NGF, TrkA and Rap1 are trafficked to early endosomes are poorly defined. We investigated trafficking and signaling of NGF, TrkA and Rap1 in PC12 cells and in cultured rat dorsal root ganglion (DRG) neurons. Herein, we show a role for both microtubule- and dynein-based transport in NGF signaling through MAPK1/2. NGF treatment resulted in trafficking of NGF, TrkA and Rap1 to early endosomes in the perinuclear region of PC12 cells where sustained activation of MAPK1/2 was observed. Disruption of microtubules with nocodazole in PC12 cells had no effect on the activation of TrkA and Ras. However, it disrupted intracellular trafficking of TrkA and Rap1. Moreover, NGF-induced activation of Rap1 and sustained activation of MAPK1/2 were markedly suppressed. Inhibition of dynein activity through overexpression of dynamitin (p50) blocked trafficking of Rap1 and the sustained phase of MAPK1/2 activation in PC12 cells. Remarkably, even in the continued presence of NGF, mature DRG neurons that overexpressed p50 became atrophic and most (>80%) developing DRG neurons died. Dynein- and microtubule-based transport is thus necessary for TrkA signaling to Rap1 and MAPK1/2.

3.994 **Angiotensin II Decreases Glucose Uptake by Downregulation of GLUT1 in the Cell Membrane of the Vascular Smooth Muscle Cell Line A10**
Masori, M. et al
J. Cardiovasc. Pharmacol., ***50(3)***, *267-273 (2007)*

Recent evidence suggests a crosstalk between angiotensin II (Ang II) and insulin. However, whether this crosstalk affects glucose uptake, particularly in terms of actin filament involvement, has not yet been studied in vascular smooth muscle cells. Pretreatment of cells with either Ang II or cytochalasin D disarranged actin filaments in a time-dependent manner and inhibited glucose uptake. However, insulin increased actin reorganization and glucose uptake. Membrane fractionation studies showed that Ang II decreased GLUT-1 at the cell membrane, whereas it increased GLUT-1 in the cytoplasm, indicating that Ang II may cause internalization of GLUT-1 via actin disorganization, consequently decreasing glucose uptake. The effects of Ang II on glucose uptake and actin reorganization were blocked by AT1 receptor antagonist, but not by AT2 antagonist. Either P38 or ERK1/2 inhibitors partially reversed the Ang II-

inhibited actin reorganization and glucose uptake, suggesting that MAPK signaling pathways could be involved as downstream events in Ang II signaling, and this signaling may interfere with insulin-induced actin reorganization and glucose uptake. These data imply that Ang II induces insulin resistance by decreasing glucose uptake via disarrangement of actin filaments, which provides a novel insight into understanding of insulin resistance by Ang II at the molecular level.

3.995 BK Channels Are Linked to Inositol 1,4,5-Triphosphate Receptors via Lipid Rafts: A NOVEL MECHANISM FOR COUPLING [Ca2+]i TO ION CHANNEL ACTIVATION
Weaver, A.K., Olsen, M.L., McFerrin, M.B. and Southeimer, H.
J. Biol. Chem., ***282(43)***, *31558-31568 (2007)*

Glioma cells prominently express a unique splice variant of a large conductance, calcium-activated potassium channel (BK channel). These channels transduce changes in intracellular calcium to changes of K^+ conductance in the cells and have been implicated in growth control of normal and malignant cells. The Ca^{2+} increase that facilitates channel activation is thought to occur via activation of intracellular calcium release pathways or influx of calcium through Ca^{2+}-permeable ion channels. We show here that BK channel activation involves the activation of inositol 1,4,5-triphosphate receptors (IP_3R), which localize near BK channels in specialized membrane domains called lipid rafts. Disruption of lipid rafts with methyl-β-cyclodextrin disrupts the functional association of BK channel and calcium source resulting in a >50% reduction in K^+ conductance mediated by BK channels. The reduction of BK current by lipid raft disruption was overcome by the global elevation of intracellular calcium through inclusion of 750 nM Ca^{2+} in the pipette solution, indicating that neither the calcium sensitivity of the channel nor their overall number was altered. Additionally, pretreatment of glioma cells with 2-aminoethoxydiphenyl borate to inhibit IP_3Rs negated the effect of methyl-β-cyclodextrin, providing further support that IP_3Rs are the calcium source for BK channels. Taken together, these data suggest a privileged association of BK channels in lipid raft domains and provide evidence for a novel coupling of these Ca^{2+}-sensitive channels to their second messenger source.

3.996 Escherichia coli Signal Recognition Particle Receptor FtsY Contains an Essential and Autonomous Membrane-binding Amphipathic Helix
Parlitz, R. et al
J. Biol. Chem., ***282(44)***, *32176-32184 (2007)*

Escherichia coli membrane protein biogenesis is mediated by a signal recognition particle and its membrane-associated receptor (FtsY). Although crucial for its function, it is still not clear how FtsY interacts with the membrane. Analysis of the structure/function differences between severely truncated active (NG+1) and inactive (NG) mutants of FtsY enabled us to identify an essential membrane-interacting determinant. Comparison of the three-dimensional structures of the mutants, combined with site-directed mutagenesis, modeling, and liposome-binding assays, revealed that FtsY contains a conserved autonomous lipid-binding amphipathic α-helix at the N-terminal end of the N domain. Deletion experiments showed that this helix is essential for FtsY function *in vivo*, thus offering, for the first time, clear evidence for the functionally important, physiologically relevant interaction of FtsY with lipids.

3.997 Saccharomyces cerevisiae CWH43 Is Involved in the Remodeling of the Lipid Moiety of GPI Anchors to Ceramides
Umemura, M., Fujita, M., Yoko-o., T., Fukamizu, A. and Jigami, Y.
Mol. Biol. Cell, ***18***, *4304-4316 (2007)*

The glycosylphosphatidylinositol (GPI)-anchored proteins are subjected to lipid remodeling during their biosynthesis. In the yeast *Saccharomyces cerevisiae*, the mature GPI-anchored proteins contain mainly ceramide or diacylglycerol with a saturated long-fatty acid, whereas conventional phosphatidylinositol (PI) used for GPI biosynthesis contains an unsaturated fatty acid. Here, we report that *S. cerevisiae* Cwh43p, whose N-terminal region contains a sequence homologous to mammalian PGAP2, is involved in the remodeling of the lipid moiety of GPI anchors to ceramides. In *cwh43* disruptant cells, the PI moiety of the GPI-anchored protein contains a saturated long fatty acid and lyso-PI but not inositolphosphorylceramides, which are the main lipid moieties of GPI-anchored proteins from wild-type cells. Moreover, the C-terminal region of Cwh43p (Cwh43-C), which is not present in PGAP2, is essential for the ability to remodel GPI lipids to ceramides. The N-terminal region of Cwh43p (Cwh43-N) is associated with Cwh43-C, and it enhanced the lipid remodeling to ceramides by Cwh43-C. Our results also indicate that mouse FRAG1 and C130090K23, which are homologous to Cwh43-N and -C, respectively, share these activities.

3.998 A novel beta-site amyloid precursor protein cleaving enzyme (BACE) isoform regulated by nonsense-mediated mRNA decay and proteasome-dependent degradation
Tanahashi, H. and Tabira, T.
Neurosci. Lett., ***428***, *103-108 (2007)*

Proteolytic cleavage of amyloid beta-peptide (Aβ) from amyloid precursor protein (APP) is a key event in the pathogenesis of Alzheimer's disease. Beta-site amyloid precursor protein cleaving enzyme (BACE) cleaves the APP at the N-terminus of Aβ. We investigated whether particular stress conditions modify the expression and activity of BACE, and found that treatment of human neuroblastoma cells with protein synthesis inhibitors induced expression of a novel splice variant of BACE. This unusual transcript, I-127, is produced by usage of an internal splicing donor site in exon 3. The splicing event leads to a premature termination codon, as well as elimination of one of two conserved aspartic protease active sites, a transmembrane domain, and a C-terminal cytoplasmic tail from BACE. Low levels of this mRNA were found in the human brain. When expressed in cells, I-127 had no effect on Aβ secretion and was retained in the endoplasmic reticulum without propeptide removal. It was also unstable with a turnover $t_{1/2}$ of ~ 2 h; normal BACE had a turnover $t_{1/2}$ of ~8 h. Finally, I-127 was degraded in a proteasome-dependent manner. Thus, I-127 is regulated by both nonsense-mediated mRNA decay (NMD) and proteasome-dependent degradation.

3.999 Plant organelle proteomics
Lilley, K.S. and Dupree, P.
Current Opinion in Plant Biol., ***10***, *594-599 (2007)*

It is important for cell biologists to know the subcellular localization of proteins to understand fully the functions of organelles and the compartmentation of plant metabolism. The accurate description of an organelle proteome requires the ability to identify genuine protein residents. Such accurate assignment is difficult in situations where a pure homogeneous preparation of the organelle cannot be achieved. Practical limitations in both organelle isolation and also analysis of low abundance proteins have resulted in limited datasets from high throughput proteomics approaches. Here, we discuss some examples of quantitative proteomic methods and their use to study plant organelle proteomes, with particular reference to methods designed to give unequivocal assignments to organelles.

3.1000 Comparison of the contributions of the nuclear and cytoplasmic compartments to global gene expression in human cells
Barthelson, R.A., Lambert, G.M., Vanier, C., Lynch, R.M. and Galbraith, D.W.
BMC Genomics, ***8(340),*** *1-15 (2007)*

Background
In the most general sense, studies involving global analysis of gene expression aim to provide a comprehensive catalog of the components involved in the production of recognizable cellular phenotypes. These studies are often limited by the available technologies. One technology, based on microarrays, categorizes gene expression in terms of the abundance of RNA transcripts, and typically employs RNA prepared from whole cells, where cytoplasmic RNA predominates.
Results
Using microarrays comprising oligonucleotide probes that represent either protein-coding transcripts or microRNAs (miRNA), we have studied global transcript accumulation patterns for the HepG2 (human hepatoma) cell line. Through subdividing the total pool of RNA transcripts into samples from nuclei, the cytoplasm, and whole cells, we determined the degree of correlation of these patterns across these different subcellular locations. The transcript and miRNA abundance patterns for the three RNA fractions were largely similar, but with some exceptions: nuclear RNA samples were enriched with respect to the cytoplasm in transcripts encoding proteins associated with specific nuclear functions, such as the cell cycle, mitosis, and transcription. The cytoplasmic RNA fraction also was enriched, when compared to the nucleus, in transcripts for proteins related to specific nuclear functions, including the cell cycle, DNA replication, and DNA repair. Some transcripts related to the ubiquitin cycle, and transcripts for various membrane proteins were sorted into either the nuclear or cytoplasmic fractions.
Conclusion
Enrichment or compartmentalization of cell cycle and ubiquitin cycle transcripts within the nucleus may be related to the regulation of their expression, by preventing their translation to proteins. In this way, these cellular functions may be tightly controlled by regulating the release of mRNA from the nucleus and

thereby the expression of key rate limiting steps in these pathways. Many miRNA precursors were also enriched in the nuclear samples, with significantly fewer being enriched in the cytoplasm. Studies of mRNA localization will help to clarify the roles RNA processing and transport play in the regulation of cellular function.

3.1001 Integral and Associated Lysosomal Membrane Proteins
Schröder, B. et al
Traffic, ***8(12)***, *1676-1686 (2007)*

We searched for novel proteins in lysosomal membranes, tentatively participating in molecular transport across the membrane and/or in interactions with other compartments. In membranes purified from placental lysosomes, we identified 58 proteins, known to reside at least partially in the lysosomal membrane. These included 17 polypeptides comprising or associated with the vacuolar adenosine triphosphatase. We report on additional 86 proteins that were significantly enriched in the lysosomal membrane fraction. Among these, 12 novel proteins of unknown functions were found. Three were orthologues of rat proteins that have been identified in tritosomes by Bagshaw RD et al. (A proteomic analysis of lysosomal integral membrane proteins reveals the diverse composition of the organelle. Mol Cell Proteomics 2005;4:133–143). Here, the proteins encoded by LOC201931 (FLJ38482) and LOC51622 (C7orf28A) were expressed with an appended fluorescent tag in HeLa cells and found to be present in lysosomal organelles. Among the lysosomally enriched proteins, also 16 enzymes and transporters were detected that had not been assigned to lysosomal membranes previously. Finally, our results identified a particular set of proteins with known functions in signaling and targeting to be at least partially associated with lysosomes.

3.1002 Function and dynamics of PKD2 in Chlamydomonas reinhardtii flagella
Huang, K. et al
J. Cell Biol., ***179(3)***, *501-514 (2007)*

To analyze the function of ciliary polycystic kidney disease 2 (PKD2) and its relationship to intraflagellar transport (IFT), we cloned the gene encoding *Chlamydomonas reinhardtii* PKD2 (CrPKD2), a protein with the characteristics of PKD2 family members. Three forms of this protein (210, 120, and 90 kD) were detected in whole cells; the two smaller forms are cleavage products of the 210-kD protein and were the predominant forms in flagella. In cells expressing CrPKD2-GFP, about 10% of flagellar CrPKD2-GFP was observed moving in the flagellar membrane. When IFT was blocked, fluorescence recovery after photobleaching of flagellar CrPKD2-GFP was attenuated and CrPKD2 accumulated in the flagella. Flagellar CrPKD2 increased fourfold during gametogenesis, and several *CrPKD2* RNA interference strains showed defects in flagella-dependent mating. These results suggest that the CrPKD2 cation channel is involved in coupling flagellar adhesion at the beginning of mating to the increase in flagellar calcium required for subsequent steps in mating.

3.1003 SorLA/LR11 Regulates Processing of Amyloid Precursor Protein via Interaction with Adaptors GGA and PACS-1
Schmidt, V. et al
J. Biol. Chem., ***282(45)***, *32956-32964 (2007)*

SorLA has been recognized as a novel sorting receptor that regulates trafficking and processing of the amyloid precursor protein (APP) and that represents a significant risk factor for sporadic Alzheimer disease. Here, we investigated the cellular mechanisms that control intracellular trafficking of sorLA and their relevance for APP processing. We demonstrate that sorLA acts as a retention factor for APP in trans-Golgi compartments/trans-Golgi network, preventing release of the precursor into regular processing pathways. Proper localization and activity of sorLA are dependent on functional interaction with GGA and PACS-1, adaptor proteins involved in protein transport to and from the trans-Golgi network. Aberrant targeting of sorLA to the recycling compartment or the plasma membrane causes faulty APP trafficking and imbalance in non-amyloidogenic and amyloidogenic processing fates. Thus, our findings identified altered routing of sorLA as a major cellular mechanism contributing to abnormal APP processing and enhanced amyloid β-peptide formation.

3.1004 Myristoylation Is Required for Human Immunodeficiency Virus Type 1 Gag-Gag Multimerization in Mammalian Cells
Li, H., Dou, J., Ding, L. and Spearman, P.

J. Virol., ***81(23)***, *12899-12910 (2007)*

The Gag protein of human immunodeficiency virus type 1 directs the virion assembly process. Gag proteins must extensively multimerize during the formation of the spherical immature virion shell. In vitro, virus-like particles can be generated from Gag proteins that lack the N-terminal myristic acid modification or the nucleocapsid (NC) protein. The precise requirements for Gag-Gag multimerization under conditions present in mammalian cells, however, have not been fully elucidated. In this study, a Gag-Gag multimerization assay measuring fluorescence resonance energy transfer was employed to define the Gag domains that are essential for homomultimerization. Three essential components were identified: protein-protein interactions contributed by residues within both the N- and C-terminal domains of capsid (CA), basic residues in NC, and the presence of myristic acid. The requirement of myristic acid for multimerization was reproduced using the heterologous myristoylation sequence from v-*src*. Only when a leucine zipper dimerization motif was placed in the position of NC was a nonmyristoylated Gag protein able to multimerize. These results support a three-component model for Gag-Gag multimerization that includes membrane interactions mediated by the myristoylated N terminus of Gag, protein-protein interactions between CA domains, and NC-RNA interactions.

3.1005 Induction of Epidermal Growth Factor Receptor Expression by Epstein-Barr Virus Latent Membrane Protein 1 C-Terminal-Activating Region 1 Is Mediated by NF-κB p50 Homodimer/Bcl-3 Complexes

Thornburg, N.J. and Raab-Traub, N.
J. Virol., ***81(23)***, *12954-12961 (2007)*

The Epstein-Barr virus (EBV) is associated with the development of numerous malignancies, including the epithelial malignancy nasopharyngeal carcinoma (NPC). The viral oncoprotein latent membrane protein 1 (LMP1) is expressed in almost all EBV-associated malignancies and has profound effects on gene expression. LMP1 acts as a constitutively active tumor necrosis factor receptor and activates multiple forms of the NF-κB family of transcription factors. LMP1 has two domains that both activate NF-κB. In epithelial cells, LMP1 C-terminal activating region 1 (CTAR1) uniquely activates p50/p50-, p50/p52-, and p65-containing complexes while CTAR2 activates canonical p50/p65 complexes. CTAR1 also uniquely upregulates the epidermal growth factor receptor (EGFR). In NPC, NF-κB p50/p50 homodimers and the transactivator Bcl-3 were detected on the EGFR promoter. In this study, the role of NF-κB p50 and Bcl-3 in LMP1-mediated upregulation of EGFR was analyzed. In LMP1-CTAR1-expressing cells, chromatin immunoprecipitation detected p50 and Bcl-3 on the NF-κB consensus sites within the *egfr* promoter. Transient overexpression of p50 and Bcl-3 increased EGFR expression, confirming the regulation of EGFR by these factors. Treatment with p105/p50 siRNA effectively reduced p105/p50 levels but unexpectedly increased Bcl-3 expression and levels of p50/Bcl-3 complexes, resulting in increased EGFR expression. These data suggest that induction of p50/p50/Bcl-3 complexes by LMP1 CTAR1 mediates LMP1-induced EGFR upregulation and that formation of the p50/p50/Bcl-3 complex is negatively regulated by the p105 precursor. The distinct forms of NF-κB that are induced by LMP1 CTAR1 likely activate distinct cellular genes.

3.1006 Human Immunodeficiency Virus Type 1 Nef protein modulates the lipid composition of virions and host cell membrane microdomains

Brügger, B. et al
Retrovirology, ***4(70)***, *1-12*

Background
The Nef protein of Human Immunodeficiency Viruses optimizes viral spread in the infected host by manipulating cellular transport and signal transduction machineries. Nef also boosts the infectivity of HIV particles by an unknown mechanism. Recent studies suggested a correlation between the association of Nef with lipid raft microdomains and its positive effects on virion infectivity. Furthermore, the lipidome analysis of HIV-1 particles revealed a marked enrichment of classical raft lipids and thus identified HIV-1 virions as an example for naturally occurring membrane microdomains. Since Nef modulates the protein composition and function of membrane microdomains we tested here if Nef also has the propensity to alter microdomain lipid composition.
Results
Quantitative mass spectrometric lipidome analysis of highly purified HIV-1 particles revealed that the presence of Nef during virus production from T lymphocytes enforced their raft character via a significant reduction of polyunsaturated phosphatidylcholine species and a specific enrichment of sphingomyelin. In

contrast, Nef did not significantly affect virion levels of phosphoglycerolipids or cholesterol. The observed alterations in virion lipid composition were insufficient to mediate Nef's effect on particle infectivity and Nef augmented virion infectivity independently of whether virus entry was targeted to or excluded from membrane microdomains. However, altered lipid compositions similar to those observed in virions were also detected in detergent-resistant membrane preparations of virus producing cells.
Conclusion
Nef alters not only the proteome but also the lipid composition of host cell microdomains. This novel activity represents a previously unrecognized mechanism by which Nef could manipulate HIV-1 target cells to facilitate virus propagation in vivo.

3.1007 Caveolins and intracellular calcium regulation in human airway smooth muscle
Prakash, Y.S. et al
Am. J. Physiol. Lung Cell Mol. Physiol., ***293***, *L1118-L1126 (2007)*

Regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key factor in airway smooth muscle (ASM) tone. In vascular smooth muscle, specialized membrane microdomains (caveolae) expressing the scaffolding protein caveolin-1 are thought to facilitate cellular signal transduction. In human ASM cells, we tested the hypothesis that caveolae mediate Ca^{2+} responses to agonist stimulation. Fluorescence immunocytochemistry with confocal microscopy, as well as Western blot analysis, was used to determine that agonist receptors (M_3 muscarinic, bradykinin, and histamine) and store-operated Ca^{2+} entry (SOCE)-regulatory mechanisms colocalize with caveolin-1. Although caveolin-2 coexpressed with caveolin-1, caveolin-3 was absent. In fura 2-loaded ASM cells, $[Ca^{2+}]_i$ responses to 1 μM ACh, 10 μM histamine, and 10 nM bradykinin, as well as SOCE, were attenuated (each to a different extent) after disruption of caveolae by the cholesterol-chelating drug methyl-β-cyclodextrin. Transfection of ASM cells with 50 nM caveolin-1 small interfering RNA significantly weakened caveolin-1 expression and blunted $[Ca^{2+}]_i$ responses to bradykinin and histamine, as well as SOCE, but the response to ACh was less intense. These results indicate that caveolae are present in ASM and that caveolin-1 contributes to regulation of $[Ca^{2+}]_i$ responses to agonist.

3.1008 Mitochondrial P450-dependent arachidonic acid metabolism by TCDD-induced hepatic CYP1A5; conversion of EETs to DHETs by mitochondrial soluble epoxide hydrolase
Labitzke, E.M., Diani-Moore, S. and Rifkind, A.B.
Arch. Biochem. Biophys., ***468***, *70-81 (2007)*

Several P450 enzymes localized in the endoplasmic reticulum and thought to be involved primarily in xenobiotic metabolism, including mouse and rat CYP1A1 and mouse CYP1A2, have also been found to translocate to mitochondria. We report here that the environmental toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces enzymatically active CYP1A4/1A5, the avian orthologs of mammalian CYP1A1/1A2, in chick embryo liver mitochondria as well as in microsomes. P450 proteins and activity levels (CYP1A4-dependent 7-ethoxyresorufin-*O*-deethylase and CYP1A5-dependent arachidonic acid epoxygenation) in mitochondria were 23–40% of those in microsomes. DHET formation by mitochondria was twice that of microsomes and was attributable to a mitochondrial soluble epoxide hydrolase as confirmed by Western blotting with antiEPHX2, conversion by mitochondria of pure 11,12 and 14,15-EET to the corresponding DHETs and inhibition of DHET formation by the soluble epoxide hydrolase inhibitor, 12(-3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA). TCDD also suppressed formation of mitochondrial and microsomal 20-HETE. The findings newly identify mitochondria as a site of P450-dependent arachidonic acid metabolism and as a potential target for TCDD effects. They also demonstrate that mitochondria contain soluble epoxide hydrolase and underscore a role for CYP1A in endobiotic metabolism.

3.1009 Improved subcellular fractionation of the heavy mitochondria pellet using Free Flow Electrophoresis system
Aboldzade-Bavil, A. et al
Mitochondrion, ***7****, 419 (2007)*

The preparative dissection of cells into their substructure reduces sample complexity and facilitates functional analysis of proteins in a physiological context. According to the high morphological variability of many subcellular compartments, however, cells inhabit different organellar subpopulations supposed to be linked to specialized biological functions or developmental stages. A prerequisite for a fundamental

characterization of these organellar subpopulations on the proteome level are highly pure fractions, which could not be obtained by classical separation technologies. We developed a workflow for the subcellular fractionation of the heavy mitochondria pellet using the BD Free Flow Electrophoresis system (FFE). The FFE methodology relies on the net charge of the organelles to be separated, which is caused by protein domains extending from the membrane surface. According to charge and size, particles are deflected differently in an electric field and are separated through a buffer flow perpendicular to the electric field. With this technique a second dimension separation step, relying on physical parameters not utilized in centrifugation techniques, was added to the workflow. Successful isolations of various cellular structures have been obtained by the use of this workflow. For this reason we invented FFE as an additional purification step to purify a 2.860 g fraction of rat liver tissue which mainly contained heavy mitochondria but also peroxisomes and lysosomes, so called ''high density peroxisomes and lysosomes''. The resulting pellet was loaded on an Optiprep gradient. Thereafter, each density gradient fraction was subjected to FFE. Using this workflow we were able to purify various subcellular fractions. To validate the purity and integrity of sub-fractions we performed enzyme assays for organellar markers and immunoblots with specific antibodies as well as electron microscopy. The subcellular fractionation using the FFE-system indicated that the purification was selective on the organelle level documenting the suitability of this separation technique in proteomic research.

3.1010 Identification of SVIP as an Endogenous Inhibitor of Endoplasmic Reticulum-associated Degradation

Ballar, P. et al

J. Biol. Chem., ***282(47)***, *33908-33914 (2007)*

Misfolded proteins in the endoplasmic reticulum (ER) are eliminated by a process known as ER-associated degradation (ERAD), which starts with misfolded protein recognition, followed by ubiquitination, retrotranslocation to the cytosol, deglycosylation, and targeting to the proteasome for degradation. Actions of multisubunit protein machineries in the ER membrane integrate these steps. We hypothesized that regulation of the multisubunit machinery assembly is a mechanism by which ERAD activity is regulated. To test this hypothesis, we investigated the potential regulatory role of the small p97/VCP-interacting protein (SVIP) on the formation of the ERAD machinery that includes ubiquitin ligase gp78, AAA ATPase p97/VCP, and the putative channel Derlin1. We found that SVIP is anchored to microsomal membrane via myristoylation and co-fractionated with gp78, Derlin1, p97/VCP, and calnexin to the ER. Like gp78, SVIP also physically interacts with p97/VCP and Derlin1. Overexpression of SVIP blocks unassembled CD3δ from association with gp78 and p97/VCP, which is accompanied by decreases in CD3δ ubiquitination and degradation. Silencing SVIP expression markedly enhances the formation of gp78-p97/VCP-Derlin1 complex, which correlates with increased degradation of CD3δ and misfolded Z variant of α-1-antitrypsin, established substrates of gp78. These results suggest that SVIP is an endogenous inhibitor of ERAD that acts through regulating the assembly of the gp78-p97/VCP-Derlin1 complex.

3.1011 The Confluence-dependent Interaction of Cytosolic Phospholipase A2-α with Annexin A1 Regulates Endothelial Cell Prostaglandin E2 Generation

Herbert, S.P., Odell, A.F., Ponnambalam, S. and walker, J.H.

J. Biol. Chem., ***282(47)***, *3468-3478 (2007)*

The regulated generation of prostaglandins from endothelial cells is critical to vascular function. Here we identify a novel mechanism for the regulation of endothelial cell prostaglandin generation. Cytosolic phospholipase A_2-α (cPLA$_2\alpha$) cleaves phospholipids in a Ca^{2+}-dependent manner to yield free arachidonic acid and lysophospholipid. Arachidonic acid is then converted into prostaglandins by the action of cyclooxygenase enzymes and downstream synthases. By previously undefined mechanisms, nonconfluent endothelial cells generate greater levels of prostaglandins than confluent cells. Here we demonstrate that Ca^{2+}-independent association of cPLA$_2\alpha$ with the Golgi apparatus of confluent endothelial cells correlates with decreased prostaglandin synthesis. Golgi association blocks arachidonic acid release and prevents functional coupling between cPLA$_2\alpha$ and COX-mediated prostaglandin synthesis. When inactivated at the Golgi apparatus of confluent endothelial cells, cPLA$_2\alpha$ is associated with the phospholipid-binding protein annexin A1. Furthermore, the siRNA-mediated knockdown of endogenous annexin A1 significantly reverses the inhibitory effect of confluence on endothelial cell prostaglandin generation. Thus the confluence-dependent interaction of cPLA$_2\alpha$ and annexin A1 at the Golgi acts as a novel molecular switch controlling cPLA$_2\alpha$ activity and endothelial cell prostaglandin generation.

3.1012 **The Nuclear RhoA Exchange Factor Net1 Interacts with Proteins of the Dlg Family, Affects Their Localization, and Influences Their Tumor Suppressor Activity**
Garcia-Mata, R. et al
Mol. Cell. Biol., ***27(24)***, *8683-8697 (2007)*

Net1 is a RhoA-specific guanine nucleotide exchange factor which localizes to the nucleus at steady state. A deletion in its N terminus redistributes the protein to the cytosol, where it activates RhoA and can promote transformation. Net1 contains a PDZ-binding motif at the C terminus which is essential for its transformation properties. Here, we found that Net1 interacts through its PDZ-binding motif with tumor suppressor proteins of the Dlg family, including Dlg1/SAP97, SAP102, and PSD95. The interaction between Net1 and its PDZ partners promotes the translocation of the PDZ proteins to nuclear subdomains associated with PML bodies. Interestingly, the oncogenic mutant of Net1 is unable to shuttle the PDZ proteins to the nucleus, although these proteins still associate as clusters in the cytosol. Our results suggest that the ability of oncogenic Net1 to transform cells may be in part related to its ability to sequester tumor suppressor proteins like Dlg1 in the cytosol, thereby interfering with their normal cellular function. In agreement with this, the transformation potential of oncogenic Net1 is reduced when it is coexpressed with Dlg1 or SAP102. Together, our results suggest that the interaction between Net1 and Dlg1 may contribute to the mechanism of Net1-mediated transformation.

3.1013 **Endocytic Trafficking of Sphingomyelin Depends on Its Acyl Chain Length**
Koivusalo, M., Jansen, M., Somerharju, P and Ikonen, E.
Mol. Biol. Cell, ***18***, *5113-5123 (2007)*

To study the principles of endocytic lipid trafficking, we introduced pyrene sphingomyelins (PyrSMs) with varying acyl chain lengths and domain partitioning properties into human fibroblasts or HeLa cells. We found that a long-chain, ordered-domain preferring PyrSM was targeted Hrs and Tsg101 dependently to late endosomal compartments and recycled to the plasma membrane in an NPC1- and cholesterol-dependent manner. A short-chain, disordered domain preferring PyrSM recycled more effectively, by using Hrs-, Tsg101- and NPC1-independent routing that was insensitive to cholesterol loading. Similar chain length-dependent recycling was observed for unlabeled sphingomyelins (SMs). The findings 1) establish acyl chain length as an important determinant in the endocytic trafficking of SMs, 2) implicate ESCRT complex proteins and NPC1 in the endocytic recycling of ordered domain lipids to the plasma membrane, and 3) introduce long-chain PyrSM as the first fluorescent lipid tracing this pathway.

3.1014 **Tight junctions contain oligomeric protein assembly critical for maintaining blood–brain barrier integrity in vivo**
McCaffrey, G. et al
J. Neurochem., ***103***, *2540-2555 (2007)*

Tight junctions (TJs) are major components of the blood–brain barrier (BBB) that physically obstruct the interendothelial space and restrict paracellular diffusion of blood-borne substances from the peripheral circulation to the CNS. TJs are dynamic structures whose intricate arrangement of oligomeric transmembrane and accessory proteins rapidly alters in response to external stressors to produce changes in BBB permeability. In this study, we investigate the constitutive trafficking of the TJ transmembrane proteins occludin and claudin-5 that are essential for forming the TJ seal between microvascular endothelial cells that inhibits paracellular diffusion. Using a novel, detergent-free **OptiPrep** density-gradient method to fractionate rat cerebral microvessels, we identify a plasma membrane lipid raft domain that contains oligomeric occludin and claudin-5. Our data suggest that oligomerization of occludin involves disulfide bond formation within transmembrane regions, and that assembly of the TJ oligomeric protein complex is facilitated by an oligomeric caveolin scaffold. This is the first time that distribution of oligomeric TJ transmembrane proteins within plasma membrane lipid rafts at the BBB has been examined *in vivo*. The findings reported in this study are critical to understand the mechanism of assembly of the TJ multiprotein complex that is essential for maintaining BBB integrity.

3.1015 **Adaptor Protein LAPF Recruits Phosphorylated p53 to Lysosomes and Triggers Lysosomal Destabilization in Apoptosis**
Li, N. et al
CancerRes., ***67(23)***, *11176-11185 (2007)*

Evidence suggests a functional association between the tumor suppressor p53 and apoptosis-involved organelle lysosome; however, the detailed mechanisms remain poorly understood. We recently reported that a lysosome-targeting protein, LAPF (lysosome-associated and apoptosis-inducing protein containing PH and FYVE domains), could initiate apoptosis of L929 cells through a lysosomal-mitochondrial pathway. In this study, we show that LAPF specifically interacted with phosphorylated p53 ($Ser^{15/18}$) both *in vitro* and *in vivo*, which could be enhanced by apoptotic stimuli, such as tumor necrosis factor α(TNF-α) and ionizing irradiation. The PH domain of LAPF and the transactivation domain of p53 mediated the interaction between both molecules. Phosphorylated p53 ($Ser^{15/18}$) could translocate to lysosomes before lysosomal membrane permeabilization (LMP) in LAPF-initiated and TNF-induced apoptosis. Silencing of LAPF expression abrogated lysosomal translocation of phosphorylated p53 ($Ser^{15/18}$), whereas silencing of p53 expression had no effect on lysosomal translocation of LAPF. Similar to that of LAPF silencing, silencing of endogenous p53 expression in L929 cells could significantly impair TNF-α–induced LMP and apoptosis. However, reexpression of wild-type p53, p53S15D (substitution of Ser^{15} to Asp that mimics a phosphorylated state), and p53R175H (a transcription-deficient mutant) in p53-knockdown L929 cells could rescue the decrease in TNF-induced apoptosis. The data suggest that phosphorylated p53 ($Ser^{15/18}$) might translocate to lysosome via forming complexes with adaptor protein LAPF and subsequently result in LMP and apoptosis, which might be in a transcription-independent manner.

3.1016 EGF-induced PIP2 hydrolysis releases and activates cofilin locally in carcinoma cells
Van Rheenen, J. et al
J. Cell Biol., ***179(6)***, *1247-1259 (2007)*

Lamellipodial protrusion and directional migration of carcinoma cells towards chemoattractants, such as epidermal growth factor (EGF), depend upon the spatial and temporal regulation of actin cytoskeleton by actin-binding proteins (ABPs). It is generally hypothesized that the activity of many ABPs are temporally and spatially regulated by PIP_2; however, this is mainly based on in vitro–binding and structural studies, and generally in vivo evidence is lacking. Here, we provide the first in vivo data that directly visualize the spatial and temporal regulation of cofilin by PIP_2 in living cells. We show that EGF induces a rapid loss of PIP_2 through PLC activity, resulting in a release and activation of a membrane-bound pool of cofilin. Upon release, we find that cofilin binds to and severs F-actin, which is coincident with actin polymerization and lamellipod formation. Moreover, our data provide evidence for how PLC is involved in the formation of protrusions in breast carcinoma cells during chemotaxis and metastasis towards EGF.

3.1017 The Epithelial Sodium Channel (ENaC) Traffics to Apical Membrane in Lipid Rafts in Mouse Cortical Collecting Duct Cells
Hill, W.G. et al
J. Biol. Chem., ***282(52)***, *37402-37411 (2007)*

We previously showed that ENaC is present in lipid rafts in A6 cells, a *Xenopus* kidney cell line. We now demonstrate that ENaC can be detected in lipid rafts in mouse cortical collecting duct ($_{MPK}CCD_{14}$) cells by detergent insolubility, buoyancy on density gradients using two distinct approaches, and colocalization with caveolin 1. Less than 30% of ENaC subunits were found in raft fractions. The channel subunits also colocalized on sucrose gradients with known vesicle targeting and fusion proteins syntaxin 1A, Vamp 2, and SNAP23. Hormonal stimulation of ENaC activity by either forskolin or aldosterone, short or long term, did not alter the lipid raft distribution of ENaC. Methyl-β-cyclodextrin added apically to $_{MPK}CCD_{14}$ cells resulted in a slow decline in amiloride-sensitive sodium transport with short circuit current reductions of 38.1 ± 9.6% after 60 min. The slow decline in ENaC activity in response to apical cyclodextrin was identical to the rate of decline seen when protein synthesis was inhibited by cycloheximide. Apical biotinylation of $_{MPK}CCD_{14}$ cells confirmed the loss of ENaC at the cell surface following cyclodextrin treatment. Acute stimulation of the recycling pool of ENaC was unaffected by apical cyclodextrin application. Expression of dominant negative caveolin isoforms (CAV1-eGFP and CAV3-DGV) which disrupt caveolae, reduced basal ENaC currents by 72.3 and 78.2%, respectively; but, as with cyclodextrin, the acute response to forskolin was unaffected. We conclude that ENaC is present in and regulated by lipid rafts. The data are consistent with a model in which rafts mediate the constitutive apical delivery of ENaC.

3.1018 Age-related subproteomic analysis of mouse liver and kidney peroxisomes
Mi, J., Garcia-Arcos, I., Alvarez, R. and Cristobal, S.
Proteome Science, ***5***, *19- (2007)*

Background

Despite major recent advances in the understanding of peroxisomal functions and how peroxisomes arise, only scant information is available regarding this organelle in cellular aging. The aim of this study was to characterize the changes in the protein expression profile of aged versus young liver and kidney peroxisome-enriched fractions from mouse and to suggest possible mechanisms underlying peroxisomal aging. Peroxisome-enriched fractions from 10 weeks, 18 months and 24 months C57bl/6J mice were analyzed by quantitative proteomics.
Results
Peroxisomal proteins were enriched by differential and density gradient centrifugation and proteins were separated by two-dimensional electrophoresis (2-DE), quantified and identified by mass spectrometry (MS). In total, sixty-five proteins were identified in both tissues. Among them, 14 proteins were differentially expressed in liver and 21 proteins in kidney. The eight proteins differentially expressed in both tissues were involved in b-oxidation, a-oxidation, isoprenoid biosynthesis, amino acid metabolism, and stress response. Quantitative proteomics, clustering methods, and prediction of transcription factors, all indicated that there is a decline in protein expression at 18 months and a recovery at 24 months.
Conclusions
These results indicate that some peroxisomal proteins show a tissue-specific functional response to aging. This response is probably dependent on their differential regeneration capacity. The differentially expressed proteins could lead several cellular effects: such as alteration of fatty acid metabolism that could alert membrane protein functions, increase of the oxidative stress and contribute to decline in bile salt synthesis. The ability to detect age-related variations in the peroxisomal proteome can help in the search for reliable and valid aging biomarkers.

3.1019 Knockdown of NHERF1 Enhances Degradation of Temperature Rescued ΔF508 CFTR from the Cell Surface of Human Airway Cells
Kwon, S-H., Pollard, H. and Giggino, W.B.
*Cell. Physiol. Biochem., **20**, 763-772 (2007)*

ΔF508 CFTR can be functionally restored in the plasma membrane by exposure of the cell to lower temperature. However, restored ΔF508 CFTR has a much shorter half-life than normal. We studied whether NHERF1, which binds to the PDZ motif of CFTR, might be a critical mediator in the turnover of ΔF508 CFTR from the cell surface. We used RNAi to reduce the expression of NHERF1 in human airway epithelial cells. Knockdown of NHERF1 reversibly reduces surface expression of WT-CFTR without altering its total expression. As expected, temperature correction increased mature C band ΔF508 CFTR (rΔF508) but unexpectedly allowed immature B band of rΔF508 to traffic to the cell surface. Both surface and total expression of rΔF508 in NHERF1 knockdown cells were reduced and degradation of surface localized rΔF508 was even faster in NHERF1 knockdown cells. Proteasomal and lysosomal inhibitor treatments led to a significant decrease in the accelerated degradation of surface rΔF508 in NHERF1 knockdown cells. These results indicate that NHERF1 plays a role in the turnover of CFTR at the cell surface, and that rΔF508 CFTR at the cell surface remains highly susceptible to degradation.

3.1020 Diversity of Raft-Like Domains in Late Endosomes
Sobo, K., Chevallier, J., Parton, R.G., Gruenberg, J. and Giscou van der Goot, F.
*PloS One, **4**, e391 (2007)*

Late endosomes, the last sorting station in the endocytic pathway before lysosomes, are pleiomorphic organelles composed of tubular elements as well as vesicular regions with a characteristic multivesicular appearance, which play a crucial role in intracellular trafficking. Here, we have investigated whether, in addition to these morphologically distinguishable regions, late endosomal membranes are additionally sub-compartmentalized into membrane microdomains.
Using sub-organellar fractionation techniques, both with and without detergents, combined with electron microscopy, we found that both the limiting membrane of the organel and the intraluminal vesicles contain raft-type membrane domains. Interestingly, these differentially localized domains vary in protein composition and physico-chemical properties.
In addition to the multivesicular organization, we find that late endosomes contain cholesterol rich microdomains both on their limiting membrane and their intraluminal vesicles that differ in composition and properties. Implications of these findings for late endosomal functions are discussed.

3.1021 Late Endosomal Cholesterol Accumulation Leads to Impaired Intra-Endosomal Trafficking
Sobo, K. et al
*PloS One, **9**, e851 (2007)*

Pathological accumulation of cholesterol in late endosomes is observed in lysosomal storage diseases such as Niemann-Pick type C. We here analyzed the effects of cholesterol accumulation in NPC cells, or as phenocopied by the drug U18666A, on late endosomes membrane organization and dynamics. Cholesterol accumulation did not lead to an increase in the raft to non-raft membrane ratio as anticipated. Strikingly, we observed a 2–3 fold increase in the size of the compartment. Most importantly, properties and dynamics of late endosomal intralumenal vesicles were altered as revealed by reduced late endosomal vacuolation induced by the mutant pore-forming toxin ASSP, reduced intoxication by the anthrax lethal toxin and inhibition of infection by the Vesicular Stomatitis Virus.
These results suggest that back fusion of intralumenal vesicles with the limiting membrane of late endosomes is dramatically perturbed upon cholesterol accumulation.

3.1022 Hsp90 Inhibition Decreases Mitochondrial Protein Turnover
Margineantu, D.H., Emerson, C.B., Diaz, D. and Hockenbery, D.M.
*PloS One, **10**, e1066 (2007)*

Background
Cells treated with hsp90 inhibitors exhibit pleiotropic changes, including an expansion of the mitochondrial compartment, accompanied by mitochondrial fragmentation and condensed mitochondrial morphology, with ultimate compromise of mitochondrial integrity and apoptosis.
Findings
We identified several mitochondrial oxidative phosphorylation complex subunits, including several encoded by mtDNA, that are upregulated by hsp90 inhibitors, without corresponding changes in mRNA abundance. Post-transcriptional accumulation of mitochondrial proteins observed with hsp90 inhibitors is also seen in cells treated with proteasome inhibitors. Detailed studies of the OSCP subunit of mitochondrial F1F0-ATPase revealed the presence of mono- and polyubiquitinated OSCP in mitochondrial fractions. We demonstrate that processed OSCP undergoes retrotranslocation to a trypsin-sensitive form associated with the outer mitochondrial membrane. Inhibition of proteasome or hsp90 function results in accumulation of both correctly targeted and retrotranslocated mitochondrial OSCP.
Conclusions
Cytosolic turnover of mitochondrial proteins demonstrates a novel connection between mitochondrial and cytosolic compartments through the ubiquitin-proteasome system. Analogous to defective protein folding in the endoplasmic reticulum, a mitochondrial unfolded protein response may play a role in the apoptotic effects of hsp90 and proteasome inhibitors.

3.1023 Kv11.1 (ERG1) K^+ Channels Localize in Cholesterol and Sphingolipid Enriched Membranes and Are Modulated by Membrane Cholesterol
Balijepalli, R.C. et al
*Channels, **14**, 263-272 (2007)*

The localization of ion channels to specific membrane microdomains can impact the functional properties of channels and their role in cellular physiology. We determined the membrane localization of human Kv11.1 (hERG1) α -subunit protein, which underlies the rapidly activating, delayed rectifier K+ current (IKr) in the heart. Immunocytochemistry and membrane fractionation using discontinuous sucrose density gradients of adult canine ventricular tissue showed that Kv11.1 channel protein localized to both the cell surface and T-tubular sarcolemma. Furthermore, density gradient membrane fractionation using detergent (Triton X-100) and non-detergent (OptiPrep) methods from canine ventricular myocytes or HEK293 cells demonstrated that Kv11.1 protein, along with MiRP1 and Kv7.1 (KCNQ1) proteins, localize in cholesterol and sphingolipid enriched membrane fractions. In HEK293 cells, Kv11.1 channels, but not long QT-associated mutant G601S-Kv11.1 channels, also localized to cholesterol and sphingolipid enriched membrane fractions. Depletion of membrane cholesterol from HEK293 cells expressing Kv11.1 channels using methyl-Mβ -cyclodextrin (Mβ CD) caused a positive shift of the voltage dependence of activation and an acceleration of deactivation kinetics of Kv11.1 current (IKv11.1). Cholesterol loading of HEK293 cells reduced the steep voltage dependence of IKv11.1 activation and accelerated the inactivation kinetics of IKv11.1. Incubation of neonatal mouse myocytes in Mβ CD also accelerated the deactivation kinetics of

IKr. We conclude that Kv11.1 protein localizes in cholesterol and sphingolipid enriched membranes and that membrane cholesterol can modulate IKv11.1 and IKr.

3.1024 Association of Botulinum Neurotoxin Serotypes A and B with Synaptic Vesicle Protein Complexes
Baldwin, M.R. and Barbieri, J.T.
Biochemistry, ***46(11)***, *3200-3210 (2007)*

Botulinum neurotoxins (BoNTs) elicit flaccid paralysis through cleavage of SNARE proteins within peripheral neurons. There are seven serotypes of the BoNTs, termed A-G, which differ in the SNARE protein and/or site that is cleaved. BoNTs are single-chain toxins that comprise an N-terminal zinc metalloprotease domain that is disulfide linked to the C-terminal translocation/receptor binding domain. SV2 and synaptotagmin have been identified as receptors for BoNT serotypes A and B, respectively. Using affinity chromatography, BoNTs A and B were observed to bind synaptic vesicle protein complexes in synaptosome lysates. Tandem LC-MS/MS identified SV2, synaptotagmin I, synaptophysin, vesicle-associated membrane protein 2 (VAMP2), and the vacuolar proton pump as components of the BoNT-receptor complex. Density gradient analysis showed that BoNT serotypes A and B exhibited unique interactions with the synaptic vesicle protein complexes. The association of BoNT serotypes A and B with synaptic vesicle protein complexes implicates a physiological role for protein complexes in synaptic vesicle biology and provides insight into the interactions of BoNT and neuronal receptors.

3.1025 Vascular Endothelial Growth Factor Receptor-3 Activity Is Modulated by Its Association with Caveolin-1 on Endothelial Membrane
Galvagni, F. et al
Biochemistry, ***46(13)***, *3998-4005 (2007)*

Vascular endothelial growth factor receptor-3 (VEGFR-3) is constitutively expressed in lymphatic vessels and transiently in endothelial cells of blood vessels during angiogenesis. Here we report that VEGFR-3 localizes in the caveolae membrane of endothelial cells and co-immunoprecipitates with caveolin-1. Caveolin-1 silencing or its depletion from the cell membrane by cholesterol increases VEGFR-3 autophosphorylation, suggesting that caveolin acts as a negative regulator of VEGFR-3 activity. Receptor activation induces caveolin-1 phosphorylation on tyrosine residues including tyrosine 14. Cell treatment with Src or Abl inhibitors PP2 or STI571, prior to receptor stimulation, affects caveolin-1 phosphorylation without affecting receptor autophosphorylation, suggesting that both Src and Abl are involved in VEGFR-3-dependent caveolin-1 phosphorylation. Caveolin-1 phosphorylation in Src/Fyn/Yes knockout cells demonstrated that Abl phosphorylates caveolin-1 independently from Src family members. These results suggest a functional interaction between VEGFR-3 and caveolin-1 to modulate endothelial cell activation during angiogenesis.

3.1026 Role of the Hydrophobic Segment of Diacylglycerol Kinase ε
Dicu, A.O., Topham, M.K., Ottaay, L. and Epand, R.M.
Biochemistry, ***46(20)***, *6109-6117 (2007)*

Diacylglycerol kinase ε(DGKε) is unique among mammalian DGK isoforms in having a segment of hydrophobic amino acids. We have evaluated the contributions of this segment to the membrane interactions and functions of this protein. To test the role of the hydrophobic segment, we have compared the properties of DGKε with those of a truncated form of the protein (DGKΔε) lacking the 40 N-terminal amino acids, which includes the hydrophobic segment. The proteins were expressed in COS-7 cells from a gene for human DGKε or from a gene for a truncated form (DGKΔε), both of which had a FLAG tag at the amino terminus. Full-length FLAG-DGKε and truncated FLAG-DGKΔε were both more specific for 1-stearoyl-2-arachidonoyl-*sn*-glycerol than for 1,2-dioleoyl-*sn*-glycerol. 1-Stearoyl-2-linoleoyl-*sn*-glycerol exhibited intermediate specificity for both forms of the enzyme. The results show that the truncated form of the enzyme maintains substrate specificity for lipids with an arachidonoyl moiety present at the *sn*-2 position. The truncation increases the catalytic rate constant for all three substrates and may suggest a role in the negative regulation of this enzyme. A full-length DGKε with a C-terminal His tag exhibited substrate specificity similar to that of the other two forms of the enzyme, indicating that the nature and position of the epitope tag did not strongly affect this property. Using an ultracentrifugation floatation assay, we showed that at neutral pH DGKΔε is extracted with 1.5 M KCl while DGKε remains essentially fully membrane bound. The full-length protein had a weak tendency to oligomerize in the presence of weak detergents. DGKε was monomeric on SDS-PAGE but exhibited partial dimerization with low concentrations of perfluorooctanoic acid. The major conclusions of this work are that the hydrophobic

domain of DGKε does not contribute to substrate specificity but plays a role in permanently sequestering the enzyme to a membrane.

3.1027 Lipid Interaction Converts Prion Protein to a PrP^{Sc}-like Proteinase K-Resistant Conformation under Physiological Conditions
Wang, F. et al
Biochemistry, ***46(23)***, *7045-7053 (2007)*

The conversion of prion protein (PrP) to the pathogenic PrP^{Sc} conformation is central to prion disease. Previous studies revealed that PrP interacts with lipids and the interaction induces PrP conformational changes, yet it remains unclear whether in the absence of any denaturing treatment, PrP-lipid interaction is sufficient to convert PrP to the classic proteinase K-resistant conformation. Using recombinant mouse PrP, we analyzed PrP-lipid interaction under physiological conditions and followed lipid-induced PrP conformational change with proteinase K (PK) digestion. We found that the PrP-lipid interaction was initiated by electrostatic contact and followed by hydrophobic interaction. The PrP-lipid interaction converted full-length α-helix-rich recombinant PrP to different forms. A significant portion of PrP gained a conformation reminiscent of PrP^{Sc}, with a PrP^{Sc}-like PK-resistant core and increased β-sheet content. The efficiency for lipid-induced PrP conversion depended on lipid headgroup structure and/or the arrangement of lipids on the surface of vesicles. When lipid vesicles were disrupted by Triton X-100, PrP aggregation was necessary to maintain the lipid-induced PrP^{Sc}-like conformation. However, the PK resistance of lipid-induced PrP^{Sc}-like conformation does not depend on amyloid fiber formation. Our results clearly revealed that the lipid interaction can overcome the energy barrier and convert full-length α-helix-rich PrP to a PrP^{Sc}-like conformation under physiological conditions, supporting the relevance of lipid-induced PrP conformational change to in vivo PrP conversion.

3.1028 Human ABCB6 Localizes to Both the Outer Mitochondrial Membrane and the Plasma Membrane
Paterson, J.K. et al
Biochemistry, ***46(33)***, *9443-9452 (2007)*

Expression of the ATP-binding cassette transporter ABCB6 has been associated with multiple cellular functions, including resistance to several cytotoxic agents, iron homeostasis, and porphyrin transport. To further elucidate its physiological function and/or role in drug resistance, we determined the subcellular location of ABCB6. Using three novel ABCB6-specific antibodies, Western blot analysis of cells expressing cDNA-derived or endogenous ABCB6 revealed two distinct molecular weight forms. Confocal microscopy indicates that the protein localizes to both mitochondria and the plasma membrane. Differential centrifugation revealed that the lower molecular weight form predominantly resides in the mitochondria, while the larger protein form is more abundant in the plasma membrane. Preliminary studies indicate that ABCB6 is functionally relevant in the plasma membrane, where its expression prevents the accumulation of specific porphyrins in the cell. Digitonin solubilization of mitochondria demonstrated that ABCB6 is present in the outer mitochondrial membrane, while back-titration assays with the ABCB6-specific antibodies reveal that the nucleotide binding domain of ABCB6 is cytoplasmic. These studies are the first to demonstrate that ABCB6 exists in two molecular weight forms, is localized to both the outer mitochondrial membrane and the plasma membrane, and plays a functional role in the plasma membrane.

3.1029 Subcellular Proteome Analysis of Camptothecin Analogue NSC606985-Treated Acute Myeloid Leukemic Cells
Yu, Y. et al
J. Proteome Res., ***6(9)***, *3808-3818 (2007)*

We reported previously that NSC606985, a camptothecin analogue, induces apoptosis of acute myeloid leukemia (AML) cells through proteolytic activation of protein kinase Cδ. Here, we analyzed protein expression profiles of fractionated nuclei, mitochondria, raw endoplasmic reticula, and cytosols of NSC606985-induced apoptotic AML cell line NB4 cells by two-dimensional electrophoresis combined with MALDI-TOF/TOF tandem mass spectrometry. In total, 90 unique deregulated proteins, including 16 compartment-compartment translocated ones, were identified. They contributed to multiple functional activities such as DNA damage repairing, chromosome assembly, mRNA processing, biosynthesis, modification, and degradation of proteins. More interestingly, several increased oxidative stress-related proteins mainly presented in mitochondria, while upregulated glycolysis proteins mainly occurred in the nuclei. With their functional analyses, the possible roles of these deregulated proteins in NSC606985-

induced apoptosis were discussed. Collectively, these discoveries would shed new insights for systematically understanding the mechanisms of the camptothecin-induced apoptosis.

3.1030 Proteomic Complex Detection Using Sedimentation
Hartman, N.T., Sicilia, F., Lilley, K.S. and Dupree, P.
Anal. Chem., ***79(5)***, *2078-2083 (2007)*

Protein-protein interactions are important in many cellular processes, but there are still relatively few methods to screen for novel protein complexes. Here we present a quantitative proteomics technique called ProCoDeS (Proteomic Complex Detection using Sedimentation) for profiling the sedimentation of a large number of proteins through a rate zonal centrifugation gradient. Proteins in a putative complex can be identified since they sediment faster than predicted from their monomer molecular weight. Using solubilized mitochondrial membrane proteins from *Arabidopsis thaliana*, the relative protein abundance in fractions of a rate zonal gradient was measured with the isotopic labeling reagent ICAT and electrospray mass spectrometry. Subunits of the same protein complex had very similar gradient distribution profiles, demonstrating the reproducibility of the quantitation method. The approximate size of the unknown complex can be inferred from its sedimentation rate relative to known protein complexes. ProCoDeS will be of use in screening extracts of tissues, cells, or organelle fractions to identify specific proteins in stable complexes that can be characterized by subsequent targeted techniques such as affinity tagging.

3.1031 PINK1 Protects against Oxidative Stress by Phosphorylating Mitochondrial Chaperone TRAP1
Pridgeon, J.W., Olzmann, J.A., Chin, L-S. and Li, L.
PloS Biology, ***5(7)***, *1494-1503 (2007)*

Mutations in the *PTEN induced putative kinase 1 (PINK1)* gene cause an autosomal recessive form of Parkinson disease (PD). So far, no substrates of PINK1 have been reported, and the mechanism by which PINK1 mutations lead to neurodegeneration is unknown. Here we report the identification of TNF receptor-associated protein 1 (TRAP1), a mitochondrial molecular chaperone also known as heat shock protein 75 (Hsp75), as a cellular substrate for PINK1 kinase. PINK1 binds and colocalizes with TRAP1 in the mitochondria and phosphorylates TRAP1 both in vitro and in vivo. We show that PINK1 protects against oxidative-stress-induced cell death by suppressing cytochrome c release from mitochondria, and this protective action of PINK1 depends on its kinase activity to phosphorylate TRAP1. Moreover, we find that the ability of PINK1 to promote TRAP1 phosphorylation and cell survival is impaired by PD-linked PINK1 G309D, L347P, and W437X mutations. Our findings suggest a novel pathway by which PINK1 phosphorylates downstream effector TRAP1 to prevent oxidative-stress-induced apoptosis and implicate the dysregulation of this mitochondrial pathway in PD pathogenesis.

3.1032 Distinct role of clathrin-mediated endocytosis in the functional uptake of cholera toxin
Vanden Broeck, D., Lagrou, A.R. and De Wolf, M.J.S.
Acta Biochimica Polonica, ***54(4)***, *757-767 (2007)*

The involvement of the clathrin-mediated endocytic internalization route in the uptake of cholera toxin (CT) was investigated using different cell lines, including the human intestinal Caco-2 and T84 cell lines, green monkey Vero cells, SH-SY5Y neuroblastoma cells and Madin-Darby canine kidney cells. Suppression of the clathrin-mediated endocytic pathway by classical biochemical procedures, like intracellular acidification and potassium depletion, inhibited cholera toxin uptake by up to about 50% as well as its ability to raise intracellular levels of cAMP. Also prior exposure of these cell types to the cationic amphiphilic drug chlorpromazine reduced the functional uptake of cholera toxin, even to a greater extent. These effects were dose- and cell type-dependent, suggesting an involvement of clathrin-mediated endocytosis in the functional uptake of cholera toxin. For a more straightforward approach to study the role of the clathrin-mediated uptake in the internalization of cholera toxin, a Caco-2^{eps-} cell line was exploited. These Caco-2^{eps-} cells constitutively suppress the expression of epsin, an essential accessory protein of clathrin-mediated endocytosis, thereby selectively blocking this internalization route. CT uptake was found to be reduced by over 60% in Caco-2^{eps-} paralleled by a diminished ability of CT to raise the level of cAMP. The data presented suggest that the clathrin-mediated uptake route fulfils an important role in the functional internalization of cholera toxin in several cell types.

3.1033 Quantitative proteomic comparison of mouse peroxisomes from liver and kidney
Mi, J., Kirchner, E. and Cristobal, S.
Proteomics, ***7(11)***, *1916-1928 (2007)*

The peroxisome plays a central role in the catabolic and anabolic pathways that contribute to the lipid homeostasis. Besides this main function, this organelle has gained functional diversity. Although several approaches have been used for peroxisomal proteome analysis, a quantitative protein expression analysis of peroxisomes from different tissues has not been elucidated yet. Here, we applied a 2-DE-based method on mouse liver and kidney peroxisomal enriched fractions to study the tissue-dependent protein expression. Ninety-one spots were identified from the 2-DE maps from pH 3.0–10.0 and 51 spots from the basic range corresponding to 31 peroxisomal proteins, 10 putative peroxisomal, 6 cytosolic, 17 mitochondrial and 1 protein from endoplasmic reticulum. Based on the identification and on the equivalent quality of both tissue preparations, the differences emerging from the comparison could be quantified. In liver, proteins involved in pathways such as α- and β-oxidation, isoprenoid biosynthesis, amino acid metabolism and purine and pirimidine metabolism were more abundant whereas in kidney, proteins from the straight-chain fatty acid β-oxidation were highly expressed. These results indicate that tissue-specific functional classes of peroxisomal proteins could be relevant to study peroxisomal cellular responses or pathologies. Finally, a web-based peroxisomal proteomic database was built.

3.1034 Analysis of the linkage of MYRIP and MYO7A to melanosomes by RAB27A in retinal pigment epithelial cells

Klomp, A.E., teofilo, K., Legacki, E. and Williams, D.S.
*Cell Motil. Cytoskeleton, **64(6)**, 474-487 (2007)*

The apical region of the retinal pigment epithelium (RPE) typically contains melanosomes. Their apical distribution is dependent on RAB27A and the unconventional myosin, MYO7A. Evidence from studies using in vitro binding assays, melanocyte transfection, and immunolocalization have indicated that the exophilin, MYRIP, links RAB27A on melanosomes to MYO7A, analogous to the manner that melanophilin links RAB27A on melanocyte melanosomes to MYO5A. To test the functionality of this hypothesis in RPE cells, we have examined the relationship among MYRIP, RAB27A and MYO7A with studies of RPE cells in primary culture (including live-cell imaging), analyses of mutant mouse retinas, and RPE cell fractionation experiments. Our results indicate that the retinal distribution of MYRIP is limited to the RPE, mainly the apical region. In RPE cells, RAB27A, MYRIP, and MYO7A were all associated with melanosomes, undergoing both slow and rapid movements. Analyses of mutant mice provide genetic evidence that MYRIP is linked to melanosomes via RAB27A, but show that recruitment of MYRIP to apical RPE is independent of melanosomes and RAB27A. RAB27A and MYRIP also associated with motile small vesicles of unknown origin. The present results provide evidence from live RPE cells that the RAB27A-MYRIP-MYO7A complex functions in melanosome motility. They also demonstrate that RAB27A provides an essential link to the melanosome.

3.1035 Reduced raft-association of NF155 in active MS-lesions is accompanied by the disruption of the paranodal junction

Maier, O., Baron, W. and Hoekstra, D.
*GLIA, **55(8)**, 885-895 (2007)*

Neurofascin155 (NF155) is required for the establishment of the paranodal axo-glial junction, the predominant interaction site between myelin and axon. It has been shown that the distribution of NF155 is altered in demyelinating diseases such as multiple sclerosis (MS). However, little is known about the biochemical mechanisms underlying these changes. We therefore compared NF155 in postmortem tissue of active and chronic inactive MS lesions with white matter from healthy controls. Although NF155 showed a very similar expression in all control white matter samples, a strong individual variation was observed in MS-lesions with NF155-levels reduced in most samples. At the same time an NF155-fragment was increased in MS-lesions, suggesting that NF155 is subject to protein degradation in lesion sites. Interestingly, the association of NF155 to membrane microdomains (rafts) was reduced in all lesions, irrespective of the amount of NF155, indicating that membrane association of NF155 was generally affected. Therefore, myelin fractionation experiments were performed to analyze the fate of paranodal proteins during demyelination. Although NF155 was enriched in heavy myelin from both control white matter and active MS-lesions, association of Caspr1/paranodin with heavy myelin was abolished in MS-lesions, demonstrating that paranodal junctions are disrupted. In conclusion, the data support the hypothesis that efficient raft-association of NF155 is essential for the assembly of the paranodal junction and demonstrate that reduced association of NF155 to lipid rafts is accompanied by the disassembly of the paranodal junction and thus contributes to the demyelination process in MS.

3.1036 Mitochondrial association of myocilin, product of a glaucoma gene, in human trabecular meshwork cells

Sakai, H., Shen, X., Koga, T., Park, B-C., Noskina, Y., Tibudan, M. and Yue, B.Y.J.T.
J. Cell. Physiol., ***213(3)***, *775-784 (2007)*

The trabecular meshwork (TM), an ocular tissue next to the cornea, is a major site for regulation of the aqueous humor outflow. Malfunctioning of this tissue is believed to be responsible for development of glaucoma, a major blinding disease. Myocilin is a gene directly linked to the most common form of glaucoma. Its protein product has been localized to both intra- and extra-cellular sites in TM cells. This study was to investigate the association of myocilin with mitochondria in TM cells. In vitro mitochondrial import assays showed that myocilin was imported to the TM mitochondria, targeting to mitochondrial membranes and/or the intermembrane space. The targeting was mediated mostly via the amino-terminal region of myocilin. When myocilin expression was induced either by treatment with dexamethasone or transfection with a myocilin construct, the mitochondrial membrane potential in TM cells, as assessed by JC-1 staining, was lowered. Subcellular fractionation and Western blot analyses confirmed that a portion of myocilin sedimented with the mitochondrial fractions. Upon anti-Fas treatment to provoke apoptosis, an increase of myocilin distribution in cytosolic fraction was observed, suggesting that myocilin was partially released from mitochondrial compartments. These results confirmed the association of myocilin with TM cell mitochondria and indicated that myocilin may have a proapoptotic role in TM cells.

3.1037 Assembly and budding of a hepatitis B virus is mediated by a novel type of intracellular vesicles

Mhamdi, M., Funk, A., Hohenberg, H., Will, H. and Sirma, H.
Hepatology, ***46(1)***, *95-106 (2007)*

Formation of enveloped viruses involves assembly and budding at cellular membranes. In this study, we elucidated the morphogenesis of hepadnaviruses on the ultrastructural and biochemical level using duck hepatitis B virus (DHBV) as a model system. Formation of virus progeny initiates at the endoplasmic reticulum (ER) and is conserved both in vitro and in vivo. The morphogenesis proceeds via membrane-surrounded vesicles containing both virions and subviral particles, indicating a common morphogenetic pathway. The virus particle–containing vesicles (VCVs) are generated and maintained by reorganization of endomembranes accompanied by a striking disorganization of the rough ER (rER). VCVs are novel organelles with unique identity and properties of ER, intermediate compartment, endosomes, and multivesicular bodies. VCVs are dynamic structures whose size and shape are regulated by both membrane fusion and fission. *Conclusion:* Our data indicate a strong reorganization of endomembranes during DHBV infection, resulting in the biogenesis of novel organelles serving as multifunctional platforms for assembly and budding of virus progeny.

3.1038 Nondetergent Isolation of Rafts

Shah, M.B. and Sehgal, P.B.
Methods Mol. Biol., ***398***, *21-28 (2007)*

Raft and caveolar microdomains have been proposed to participate in numerous cellular functions including signal transduction, cholesterol trafficking, and vesicular sorting. Traditional methods of isolation of rafts from cultured cells and tissue samples have exploited the biochemical properties of these microdomains, i.e., their relative resistance to solubilization by nonionic detergents (at 4°C) and their light buoyant density attributable to their high content of cholesterol and sphingolipids. Thus, a common way to isolate raft microdomains has been their separation on a density gradient in the presence of 0.5–1% Triton X-100 (Bochringer Mannheim Roche Applied Sciences Indianapolis, IN or Sigma-Aldrich, St. Louis, MO). This and other detergent-based methods have been discussed. However, the use of detergents may not be favorable because of artifacts that may arise with their use. (The possibility of rafts solely as detergent-induced artifacts appears to have been diffused by a number of biochemical and biophysical studies that strongly demonstrate the presence of a liquid-ordered phase within biological membranes.) In this chapter, three methods are reviewed to isolate rafts from cultured cells without the use of detergents. Two of these, the sodium carbonate and OptiPrep™ (Sigma-Aldrich St. Louis, MO) methods, are based on gradient separation and can be used to isolate rafts in general, whereas the third is a magnetic-bead immunoisolation approach and might be used to isolate subpopulations of rafts enriched for different markers such as caveolin-1, flotillin (reggie proteins), or other suitable markers. Together these methods allow for a detergent-free isolation of rafts for biochemical, proteomic, and microscopic studies.

3.1039 Proteomics-Based Method for Risk Assessment of Peroxisome Proliferating Pollutants in the Marine Environment
Cristobal, S.
Methods Mol. Biol., ***410****, 123-135 (2007)*

Pollution in aquatic environment is of increasing concern for its impact on both human and natural populations. Applying proteomics to monitor marine pollution is a new approach to evaluate the effects of environmental pollutants on the biota. Aquatic organisms living in coastal and estuarine areas are particularly prone to exposures to a variety of pollutants, some of which can act as peroxisome proliferators. However, peroxisomal responses in particular and biomarker responses in general can be influenced by several biotic and abiotic factors. Utilizing proteomics-based techniques that permit the evaluation of hundreds to thousands of proteins in a single experiment can circumvent those drawbacks. Applying this method, the peroxisomal proteome from digestive glands of mussels *Mytilus sp*. can be analyzed by two-dimensional electrophoresis (2-DE) and the 2-DE maps from control samples and samples obtained in a polluted area can be compared. The up- and down-regulated proteins compose the protein expression signature (PES) associated with exposure to peroxisome proliferating pollutants. This method generates highly reproducible patterns that can be applied to laboratory or field experiments.

3.1040 Regulation of Notch Signaling by Dynamic Changes in the Precision of S3 Cleavage of Notch-1
Tagami, S. et al
Mol. Cell. Biol., ***28(1)****, 165-176 (2008)*

Intramembrane proteolysis by presenilin-dependent γ-secretase produces the Notch intracellular cytoplasmic domain (NCID) and Alzheimer disease-associated amyloid-β. Here, we show that upon Notch signaling the intracellular domain of Notch-1 is cleaved into two distinct types of NICD species due to diversity in the site of S3 cleavage. Consistent with the N-end rule, the S3-V cleavage produces stable NICD with Val at the N terminus, whereas the S3-S/S3-L cleavage generates unstable NICD with Ser/Leu at the N terminus. Moreover, intracellular Notch signal transmission with unstable NICDs is much weaker than that with stable NICD. Importantly, the extent of endocytosis in target cells affects the relative production ratio of the two types of NICD, which changes in parallel with Notch signaling. Surprisingly, substantial amounts of unstable NICD species are generated from the Val→Gly and the Lys→Arg mutants, which have been reported to decrease S3 cleavage efficiency in cultured cells. Thus, we suggest that the existence of two distinct types of NICD points to a novel aspect of the intracellular signaling and that changes in the precision of S3 cleavage play an important role in the process of conversion from extracellular to intracellular Notch signaling.

3.1041 Protein-sphingolipid interactions within cellular membranes
Haberkant, P. et al
J. Lipid Res., ***49****, 251-262 (2008)*

Each intracellular organelle critically depends on maintaining its specific lipid composition that in turn contributes to the biophysical properties of the membrane. With our knowledge increasing about the organization of membranes with defined microdomains of different lipid compositions, questions arise regarding the molecular mechanisms that underlie the targeting to/segregation from microdomains of a given protein. In addition to specific lipid-transmembrane segment interactions as a basis for partitioning, the presence in a given microdomain may alter the conformation of proteins and, thus, the activity and availability for regulatory modifications. However, for most proteins, the specific lipid environment of transmembrane segments as well as its relevance to protein function and overall membrane organization are largely unknown. To help fill this gap, we have synthesized a novel photoactive sphingolipid precursor that, together with a precursor for phosphoglycerolipids and with photo-cholesterol, was investigated in vivo with regard to specific protein transmembrane span-lipid interactions. As a proof of principle, we show specific labeling of the ceramide transporter with the sphingolipid probe and describe specific in vivo interactions of lipids with caveolin-1, phosphatidylinositol transfer protein β, and the mature form of nicastrin. This novel photolabile sphingolipid probe allows the detection of protein-sphingolipid interactions within the membrane bilayer of living cells.

3.1042 Cytoplasmic Domain of Influenza B Virus BM2 Protein Plays Critical Roles in Production of Infectious Virus
Imai, M., Kawasaki, K. and Odagiri, T.
J. Virol., ***82(2)****, 728-739 (2008)*

Influenza B virus BM2 is a type III integral membrane protein that displays H^+ ion channel activity. Analysis of BM2 knockout mutants has suggested that this protein is a necessary component for the capture of M1-viral ribonucleoprotein (vRNP) complex at the plasma membrane and for incorporation of vRNP complex into the virion during the assembly process. BM2 comprises 109 amino acid residues and possesses a longer cytoplasmic domain than the other 3 integral membrane proteins (hemagglutinin, neuraminidase, and NB). To explore whether the cytoplasmic domain of BM2 is important for infectious virus production, a series of BM2 deletion mutants lacking three to nine amino acid residues at the carboxyl terminus, BM2Δ107-109, BM2Δ104-109, and BM2Δ101-109, was generated by reverse genetics. Intracellular transport and incorporation into virions were indistinguishable between truncated BM2 proteins and wild-type BM2. The BM2Δ107-109 mutant produced levels of infectious virus similar to those of wild-type virus and displayed a spherical shape. However, the BM2Δ104-109 and BM2Δ101-109 mutants produced viruses containing dramatically reduced vRNP complex, as with BM2 knockout mutants, and formed enlarged, irregularly shaped virions. Moreover, gradient separation of membranes indicated that membrane association of M1 from mutants was greatly affected by carboxyl-terminal truncations of BM2. Studies of alanine substitution mutants further suggested that amino acid sequences in the 98-109 region are variable while those in the 86-97 region are a prerequisite for innate BM2 function. These results indicate that the cytoplasmic domain of the BM2 protein is required for firm association of the M1 protein with lipid membranes, vRNP complex incorporation into virions, and virion morphology.

3.1043 Mint3/X11$^{\gamma}$ Is an ADP-Ribosylation Factor-dependent Adaptor that Regulates the Traffic of the Alzheimer's Precursor Protein from the Trans-Golgi Network
Shrivastava-Ranjan, P. et al
*Mol. Biol. Cell, **19**, 51-64 (2008)*

β-Amyloid peptides (Aβ) are the major component of plaques in brains of Alzheimer's patients, and are they derived from the proteolytic processing of the β-amyloid precursor protein (APP). The movement of APP between organelles is highly regulated, and it is tightly connected to its processing by secretases. We proposed previously that transport of APP within the cell is mediated in part through its sorting into Mint/X11-containing carriers. To test our hypothesis, we purified APP-containing vesicles from human neuroblastoma SH-SY5Y cells, and we showed that Mint2/3 are specifically enriched and that Mint3 and APP are present in the same vesicles. Increasing cellular APP levels increased the amounts of both APP and Mint3 in purified vesicles. Additional evidence supporting an obligate role for Mint3 in traffic of APP from the *trans*-Golgi network to the plasma membrane include the observations that depletion of Mint3 by small interference RNA (siRNA) or mutation of the Mint binding domain of APP changes the export route of APP from the basolateral to the endosomal/lysosomal sorting route. Finally, we show that increased expression of Mint3 decreased and siRNA-mediated knockdowns increased the secretion of the neurotoxic β-amyloid peptide, $A\beta_{1\text{-}40}$. Together, our data implicate Mint3 activity as a critical determinant of post-Golgi APP traffic.

3.1044 Localization of phosphorylated αB-crystallin to heart mitochondria during ischemia-reperfusion
Jin, J-K. et al
*Am. J. Physiol. Heart Circ. Physiol., **294**, H337-H344 (2008)*

The cytosolic small heat shock protein αB-crystallin (αBC) is a molecular chaperone expressed in large quantities in the heart, where it protects from stresses such as ischemia-reperfusion (I/R). Upon I/R, p38 MAP kinase activation leads to phosphorylation of αBC on Ser^{59} (P-αBC-S59), which increases its protective ability. αBC confers protection, in part, by interacting with and affecting the functions of key components in stressed cells. We investigated the hypothesis that protection from I/R damage in the heart by P-αBC-S59 can be mediated by localization to mitochondria. We found that P-αBC-S59 localized to mitochondria isolated from untreated mouse hearts and that this localization increased more than threefold when the hearts were subjected to ex vivo I/R. Mitochondrial P-αBC-S59 decreased when hearts were treated with the p38 inhibitor SB-202190. Moreover, SB-202190-treated hearts exhibited more tissue damage and less functional recovery upon reperfusion than controls. I/R activates mitochondrial permeability transition (MPT) pore opening, which increases cell damage. We found that mitochondria incubated with a recombinant mutant form of αBC that mimics P-αBC-S59 exhibited decreased calcium-induced MPT pore opening. These results indicate that mitochondria may be among the key components in stressed cells with which P-αBC-S59 interacts and that this localization may protect the myocardium, in part, by modulating MPT pore opening and, thus, reducing I/R injury.

3.1045 Lpx1p is a peroxisomal lipase required for normal peroxisome morphology
Thoms, S., Debelyy, M.O., Nau, K., Meyer, H.E. and Erdmann, R.
*FEBS J., **275**, 504-514 (2008)*

Lpx1p (systematic name: Yor084wp) is a peroxisomal protein from *Saccharomyces cerevisiae* with a peroxisomal targeting signal type 1 (PTS1) and a lipase motif. Using mass spectrometry, we have identified Lpx1p as present in peroxisomes, and show that Lpx1p import is dependent on the PTS1 receptor Pex5p. We provide evidence that Lpx1p is piggyback-transported into peroxisomes. We have expressed the Lpx1p protein in *Escherichia coli*, and show that the enzyme exerts acyl hydrolase and phospholipase A activity *in vitro*. However, the protein is not required for wild-type-like steady-state function of peroxisomes, which might be indicative of a metabolic rather than a biogenetic role. Interestingly, peroxisomes in deletion mutants of *LPX1* have an aberrant morphology characterized by intraperoxisomal vesicles or invaginations.

3.1046 Use of polarized PC12 cells to monitor protein localization in the early biosynthetic pathway
Sannerud, R., Michaël, M., Berger hansen, B. And Saraste, J.
*Methods in Mol. Biol., **457**, 253-265 (2008)*

A prerequisite for understanding the cellular functions of an unknown protein is the establishment of its subcellular localization. As increasing numbers of novel proteins of the biosynthetic pathway are currently being identified, accessible new methods are required to facilitate their localization. Differentiating rat pheochromocytoma (PC12) cells reorganize their biosynthetic membrane compartments as they develop neurite-like processes. The authors recently showed that polarization of these cells involves the expansion of the intermediate compartment (IC) between the rough endoplasmic reticulum (RER) and the Golgi apparatus. Tubules emerging from the vacuolar parts of the IC move to the developing neurites accumulating in their growth cones, whereas the vacuoles, like RER and Golgi, remain in the cell body. Thus, polarized PC12 cells enhance the resolution for immunofluorescence microscopic mapping of protein localization in the early biosynthetic pathway. The authors also describe here a rapid cell fractionation protocol employing velocity sedimentation in iodixanol gradients that allows one-step separation of the pre-Golgi vacuoles, tubules, and RER.

3.1047 Multidrug Transporters CaCdr1p and CaMdr1p of Candida albicans Display Different Lipid Specificities: both Ergosterol and Sphingolipids Are Essential for Targeting of CaCdr1p to Membrane Rafts
Pasrija, R., Panwar, S.L. and Prasad R.
*Antimicrob.Agents Chemother., **52(2)**, 694-704 (2008)*

In this study, we compared the effects of altered membrane lipid composition on the localization of two membrane drug transporters from different superfamilies of the pathogenic yeast *Candida albicans*. We demonstrated that in comparison to the major facilitator superfamily multidrug transporter CaMdr1p, ATP-binding cassette transporter CaCdr1p of *C. albicans* is preferentially localized within detergent-resistant membrane (DRM) microdomains called 'rafts.' Both CaCdr1p and CaMdr1p were overexpressed as green fluorescent protein (GFP)-tagged proteins in a heterologous host *Saccharomyces cerevisiae*, wherein either sphingolipid (Δ*sur4* or Δ*fen1* or Δ*ipt1*) or ergosterol (Δ*erg24* or Δ*erg6* or Δ*erg4*) biosynthesis was compromised. CaCdr1p-GFP, when expressed in the above mutant backgrounds, was not correctly targeted to plasma membranes (PM), which also resulted in severely impaired drug resistance. In contrast, CaMdr1p-GFP displayed no sorting defect in the mutant background and remained properly surface localized and displayed no change in drug resistance. Our data clearly show that CaCdr1p is selectively recruited, over CaMdr1p, to the DRM microdomains of the yeast PM and that any imbalance in the raft lipid constituents results in missorting of CaCdr1p.

3.1048 Compartmentalization of endocannabinoids into lipid rafts in a dorsal root ganglion cell line
Rimmermann, N. et al
*Br. J. Pharmacol., **153**, 380-389 (2008)*

Background and purpose:
N-arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG) are endogenous cannabinoids binding to the cannabinoid receptors CB_1 and CB_2 to modulate neuronal excitability and synaptic transmission in primary afferent neurons. To investigate the compartmentalization of the machinery for

AEA and 2-AG signalling, we studied their partitioning into lipid raft fractions isolated from a dorsal root ganglion X neuroblastoma cell line (F-11).
Experimental approach:
F-11 cells were homogenized and fractionated using a detergent-free OptiPrep density gradient. All lipids were partially purified from methanolic extracts of the fractions on solid phase cartridges and quantified using liquid chromatography tandem mass spectrometry (LC/MS/MS). Protein distribution was determined by Western blotting.
Key results:
Under basal conditions, the endogenous cannabinoid AEA was present in both lipid raft and specific non-lipid raft fractions as was one of its biosynthetic enzymes, NAPE-PLD. The 2-AG precursor 1-stearoyl-2-arachidonoyl-*sn*-glycerol (DAG), diacylglycerol lipase ∝(DAGL∝), which cleaves DAG to form 2-AG, and 2-AG were all co-localized with lipid raft markers. CB_1 receptors, previously reported to partition into lipid raft fractions, were not detected in F-11 membranes, but CB_2 receptors were detected at high levels and partitioned into non-lipid raft fractions.
Conclusions and implications:
The biochemical machinery for the production of 2-AG via the putative diacylglycerol pathway is localized within lipid rafts, suggesting that 2-AG synthesis via DAG occurs within these microdomains. The observed co-localization of AEA, 2-AG, and their synthetic enzymes with the reported localization of CB_1 raises the possibility of intrinsic-autocrine signalling within lipid raft domains and/or retrograde-paracrine signalling.

3.1049 The α1a-Adrenergic Receptor Occupies Membrane Rafts with Its G Protein Effectors but Internalizes via Clathrin-coated Pits
Morris, D.P., Lei, B., Wu, Y-X., Michelotti, G.-A. and Schwinn, D.A.
J. Biol. Chem., ***283(5)***, *2973-2985 (2008)*

The α_{1a}-adrenergic receptor (α_{1a}AR) occupies intracellular and plasma membranes in both native and heterologous expression systems. Based on multiple independent lines of evidence, we demonstrate the α_{1a}AR at the cell surface occupies membrane rafts but exits from rafts following stimulation. In non-detergent raft preparations, basal α_{1a}AR is present in low density membrane rafts and colocalizes with its G protein effectors on density gradients. Raft disruption by cholesterol depletion with methyl-β-cyclodextrin eliminates these light rafts. To confirm the presence of the α_{1a}AR in plasma membrane rafts, fluorescence resonance energy transfer measurements were used to demonstrate colocalization of surface receptor and the raft marker, cholera toxin B. This colocalization was largely lost following α_{1a}AR stimulation with phenylephrine. Similarly, receptor stimulation causes exit of the α_{1a}AR from light rafts within 3-10 min in contrast to the G proteins, which largely remain in light rafts. Importantly, this delayed exit of the α_{1a}AR suggests acute receptor signaling and desensitization occur entirely within rafts. Interestingly, both confocal analysis and measurement of surface α_{1a}AR levels indicate modest receptor internalization during the 10 min following stimulation, suggesting most of the receptor has entered non-raft plasma membrane. Nevertheless, activation does increase the rate of receptor internalization as does disruption of rafts with methyl-β-cyclodextrin, suggesting raft exit enables internalization. Confocal analysis of surface-labeled hemagglutinin-α_{1a}AR reveals that basal and stimulated receptor occupies clathrin pits in fixed cells consistent with previous indirect evidence. The evidence presented here strongly suggests the α_{1a}AR is a lipid raft protein under basal conditions and implies agonist-mediated signaling occurs from rafts.

3.1050 Epithelial polarity requires septin coupling of vesicle transport to polyglutamylated microtubules
Spiliotis, E.T., Hunt, S.J., Hu, Q., Kinoshita, M. And Nelson, W.J.
J. Cell Biol., ***180(2),*** *295-303 (2008)*

In epithelial cells, polarized growth and maintenance of apical and basolateral plasma membrane domains depend on protein sorting from the trans-Golgi network (TGN) and vesicle delivery to the plasma membrane. Septins are filamentous GTPases required for polarized membrane growth in budding yeast, but whether they function in epithelial polarity is unknown. Here, we show that in epithelial cells septin 2 (SEPT2) fibers colocalize with a subset of microtubule tracks composed of polyglutamylated (polyGlu) tubulin, and that vesicles containing apical or basolateral proteins exit the TGN along these SEPT2/polyGlu microtubule tracks. Tubulin-associated SEPT2 facilitates vesicle transport by maintaining polyGlu microtubule tracks and impeding tubulin binding of microtubule-associated protein 4 (MAP4). Significantly, this regulatory step is required for polarized, columnar-shaped epithelia biogenesis; upon SEPT2 depletion, cells become short and fibroblast-shaped due to intracellular accumulation of apical and basolateral membrane proteins, and loss of vertically oriented polyGlu microtubules. We suggest that

septin coupling of the microtubule cytoskeleton to post-Golgi vesicle transport is required for the morphogenesis of polarized epithelia.

3.1051 Dipeptidyl peptidase IV inhibition downregulates Na+-H+ exchanger NHE3 in rat renal proximal tubule

Castello, A. et al

Am. J. Physiol. Renal Physiol., ***294***, *F414-F422 (2008)*

In the microvillar microdomain of the kidney brush border, sodium hydrogen exchanger type 3 (NHE3) exists in physical complexes with the serine protease dipeptidyl peptidase IV (DPPIV). The purpose of this study was to explore the functional relationship between NHE3 and DPPIV in the intact proximal tubule in vivo. To this end, male Wistar rats were treated with an injection of the reversible DPPIV inhibitor Lys [$Z(NO_2)$]-pyrrolidide (I40; 60 $mg \cdot kg^{-1} \cdot day^{-1}$ ip) for 7 days. Rats injected with equal amounts of the noninhibitory compound Lys[$Z(NO_2)$]-OH served as controls. Na^+-H^+ exchange activity in isolated microvillar membrane vesicles was 45 ± 5% decreased in rats treated with I40. Membrane fractionation studies using isopycnic centrifugation revealed that I40 provoked redistribution of NHE3 along with a small fraction of DPPIV from the apical enriched microvillar membranes to the intermicrovillar microdomain of the brush border. I40 significantly increased urine output (67 ± 9%; $P < 0.01$), fractional sodium excretion (63 ± 7%; $P < 0.01$), as well as lithium clearance (81 ± 9%; $P < 0.01$), an index of end-proximal tubule delivery. Although not significant, a tendency toward decreased blood pressure and plasma pH/HCO_3^- was noted in I40-treated rats. These findings indicate that inhibition of DPPIV catalytic activity is associated with inhibition of NHE3-mediated $NaHCO_3$ reabsorption in rat renal proximal tubule. Inhibition of apical Na^+-H^+ exchange is due to reduced abundance of NHE3 protein in the microvillar microdomain of the kidney brush border. Moreover, this study demonstrates a physiologically significant interaction between NHE3 and DPPIV in the intact proximal tubule in vivo.

3.1052 Sphingolipid synthesis is necessary for kinetoplast segregation and cytokinesis in Trypanosoma brucei

Fridberg, A. et al

J. Cell Sci., ***121***, *522-535 (2008)*

Sphingolipids and their metabolites have been thought crucial for cell growth and cell cycle progression, membrane and protein trafficking, signal transduction, and formation of lipid rafts; however, recent studies in trypanosomes point to the dispensability of sphingolipids in some of these processes. In this study, we explore the requirements for de novo sphingolipid biosynthesis in the insect life cycle stage of the African trypanosome *Trypanosoma brucei* by inhibiting the enzyme serine palmitoyltransferase (SPT2) by using RNA interference or treatment with a potent SPT2 inhibitor myriocin. Mass spectrometry revealed that upon SPT2 inhibition, the parasites contained substantially reduced levels of inositolphosphorylceramide. Although phosphatidylcholine and cholesterol levels were increased to compensate for this loss, the cells were ultimately not viable. The most striking result of sphingolipid reduction in procyclic *T. brucei* was aberrant cytokinesis, characterized by incomplete cleavage-furrow formation, delayed kinetoplast segregation and emergence of cells with abnormal DNA content. Organelle replication continued despite sphingolipid depletion, indicating that sphingolipids act as second messengers regulating cellular proliferation and completion of cytokinesis. Distention of the mitochondrial membrane, formation of multilamellar structures within the mitochondrion and near the nucleus, accumulation of lipid bodies and, less commonly, disruption of the Golgi complex were observed after prolonged sphingolipid depletion. These findings suggest that some aspects of vesicular trafficking may be compromised. However, flagellar membrane targeting and the association of the flagellar membrane protein calflagin with detergent-resistant membranes were not affected, indicating that the vesicular trafficking defects were mild. Our studies indicate that sphingolipid biosynthesis is vital for cell cycle progression and cell survival, but not essential for the normal trafficking of flagellar membrane-associated proteins or lipid raft formation in procyclic *T. brucei*.

3.1053 Clustering endothelial E-selectin in clathrin-coated pits and lipid rafts enhances leukocyte adhesion under flow

Setiadi, H. and Mcever, R.P.

Blood, ***111(4)***, *1989-1998 (2008)*

During inflammation, E-selectin expressed on cytokine-activated endothelial cells mediates leukocyte rolling under flow. E-selectin undergoes endocytosis and may associate with lipid rafts. We asked whether distribution of E-selectin in membrane domains affects its functions. E-selectin was internalized in transfected CHO cells or cytokine-activated human umbilical vein endothelial cells (HUVECs). Confocal microscopy demonstrated colocalization of E-selectin with α-adaptin, a clathrin-associated protein. Deleting the cytoplasmic domain of E-selectin or disrupting clathrin-coated pits with hypertonic medium blocked internalization of E-selectin, reduced colocalization of E-selectin with α-adaptin, and inhibited E-selectin-mediated neutrophil rolling under flow. Unlike CHO cells, HUVECs expressed a small percentage of E-selectin in lipid rafts. Even fewer neutrophils rolled on E-selectin in HUVECs treated with hypertonic medium and with methyl-β-cyclodextrin, which disrupts lipid rafts. These data demonstrate that E-selectin clusters in both clathrin-coated pits and lipid rafts of endothelial cells but is internalized in clathrin-coated pits. Distribution in both domains markedly enhances E-selectin's ability to mediate leukocyte rolling under flow.

3.1054 **The Vaccinia Virus B5 Protein Requires A34 for Efficient Intracellular Trafficking from the Endoplasmic Reticulum to the Site of Wrapping and Incorporation into Progeny Virions**
Earley, A.K., Chan, W.M. and Ward, B.M.
J. Virol., ***82(5)***, *2161-2169 (2008)*

The glycoproteins encoded by the vaccinia virus A34R and B5R genes are involved in intracellular envelope virus formation and are highly conserved among orthopoxviruses. A recombinant virus that has the A34R gene deleted and the B5R gene replaced with a B5R gene fused to the enhanced green fluorescent protein (B5R-GFP) gene was created (vB5R-GFP/ΔA34R) to investigate the role of A34 during virion morphogenesis. Cells infected with vB5R-GFP/ΔA34R displayed GFP fluorescence throughout the cytoplasm, which differed markedly from that seen in cells infected with a normal B5R-GFP-expressing virus (vB5R-GFP). Immunofluorescence and subcellular fractionation demonstrated that B5-GFP localizes with the endoplasmic reticulum in the absence of A34. Expression of either full-length A34 or a construct consisting of the lumenal and transmembrane domains restored normal trafficking of B5-GFP to the site of wrapping in the juxtanuclear region. Coimmunoprecipitation studies confirmed that B5 and A34 interact through their luminal domains, and further analysis revealed that in the absence of A34, B5 is not efficiently incorporated into virions released from the cell.

3.1055 **Modulation of ileal bile acid transporter (ASBT) activity by depletion of plasma membrane cholesterol: association with lipid rafts**
Annaba, F. et al
Am. J. Physiol. Gastrointest. Liver Physiol., ***294***, *G489-G497 (2008)*

Apical sodium-dependent bile acid transporter (ASBT) represents a highly efficient conservation mechanism of bile acids via mediation of their active transport across the luminal membrane of terminal ileum. To gain insight into the cellular regulation of ASBT, we investigated the association of ASBT with cholesterol and sphingolipid-enriched specialized plasma membrane microdomains known as lipid rafts and examined the role of membrane cholesterol in maintaining ASBT function. Human embryonic kidney (HEK)-293 cells stably transfected with human ASBT, human ileal brush-border membrane vesicles, and human intestinal epithelial Caco-2 cells were utilized for these studies. Floatation experiments on **Optiprep** density gradients demonstrated the association of ASBT protein with lipid rafts. Disruption of lipid rafts by depletion of membrane cholesterol with methyl-β-cyclodextrin (MβCD) significantly reduced the association of ASBT with lipid rafts, which was paralleled by a decrease in ASBT activity in Caco-2 and HEK-293 cells treated with MβCD. The inhibition in ASBT activity by MβCD was blocked in the cells treated with MβCD-cholesterol complexes. Kinetic analysis revealed that MβCD treatment decreased the V_{max} of the transporter, which was not associated with alteration in the plasma membrane expression of ASBT. Our study illustrates that cholesterol content of lipid rafts is essential for the optimal activity of ASBT and support the association of ASBT with lipid rafts. These findings suggest a novel mechanism by which ASBT activity may be rapidly modulated by alterations in cholesterol content of plasma membrane and thus have important implications in processes related to maintenance of bile acid and cholesterol homeostasis.

3.1056 **Ceramide kinase promotes Ca2+ signaling near IgG-opsonized targets and enhances phagolysosomal fusion in COS-1 cells**
Hinkovska-Galcheva, V. et al
J. Lipid Res., ***49***, *531-542 (2008)*

Ceramide-1-phosphate (C1P) is a novel bioactive sphingolipid formed by the phosphorylation of ceramide catalyzed by ceramide kinase (CERK). In this study, we evaluated the mechanism by which increased C1P during phagocytosis enhances phagocytosis and phagolysosome formation in COS-1 cells expressing hCERK. Stable transfectants of COS-1 cells expressing FcγRIIA or both FcγRIIA/hCERK expression vectors were created. Cell fractionation studies demonstrated that hCERK and the transient receptor potential channel (TRPC-1) were enriched in caveolae fractions. Our data establish that both CERK and TRPC-1 localize to the caveolar microdomains during phagocytosis and that CERK also colocalizes with EIgG in FcγRIIA/hCERK-bearing COS-1 cells. Using high-speed fluorescence microscopy, FcγRIIA/hCERK transfected cells displayed Ca^{2+} sparks around the phagosome. In contrast, cells expressing FcγRIIA under identical conditions displayed little periphagosomal Ca^{2+} signaling. The enhanced Ca^{2+} signals were accompanied by enhanced phagolysosome formation. However, the addition of pharmacological reagents that inhibit store-operated channels (SOCs) reduced the phagocytic index and phagolysosomal fusion in hCERK transfected cells. The higher Ca^{2+} signal observed in hCERK transfected cells as well as the fact that CERK colocalized with EIgG during phagocytosis support our hypothesis that Ca^{2+} signaling is an important factor for increasing phagocytosis and is regulated by CERK in a manner that involves SOCs/TRPCs.

3.1057 Cross-talk between PDGF and S1P signalling elucidates the inhibitory effect and potential antifibrotic action of the immunomodulator FTY720 in activated HSC-cultures
Brunati, A.M. et al
*Biochim. Biophys. Acta, **1783**, 347-359 (2008)*

Platelet-derived growth factor (PDGF) has been shown to be essential in the activation of hepatic stellate cells (HSCs), contributing to the onset and development of hepatic fibrosis. Recently, sphingosine-1-phosphate (S1P) has been shown to be a mitogen and stimulator of chemotaxis also for HSCs. Since it has been demonstrated in several cell types that cross-talk between PDGF and S1P signalling pathways occurs, our aim was to investigate the potential antifibrotic effect of FTY720, whose phosphorylated form acts as a potent S1P receptor (S1PR) modulator, on HSCs. FTY720 inhibits cell proliferation and migration after PDGF stimulation on HSCs in a concentration range between 0.1 and 1 μM. By using compounds that block S1P signalling (PTX and VPC23019), we assessed that FTY720 also acts in an S1P receptor-independent way by decreasing the level of tyrosine phosphorylation of PDGF receptor, with subsequent inhibition of the PDGF signalling pathway. In addition, inhibition of sphingosine kinase2 (SphK2), which is responsible for FTY720 phosphorylation, by DMS/siRNA unveils a mechanism of action irrespective of its phosphorylation, in particular decreasing the level of $S1P_1$ on the plasma membrane. These findings led us to hypothesize a potential use of FTY720 as a potential antifibrotic drug for further clinical application.

3.1058 Lipid rafts regulate ethanol-induced activation of TLR4 signaling in murine macrophages
Fernandez-Lizarbe, S., Pascual, M., Soledad Gascon, M., Blanco, A. and Guerri, C.
*Mol. Immunol., **45**, 2007-2016 (2008)*

Toll-like receptors (TLRs) response is critical in innate resistance to infection. Alcohol consumption has been shown to suppress the inflammatory response mediated through TLR4, down regulating the production of inflammatory cytokines. We recently reported that low concentrations of ethanol activate TLR4 signaling in astrocytes and triggers neuroinflammation. Because macrophages are important cells in innate immunity, we investigate whether low concentrations of ethanol could stimulate the TLR4 signaling response in murine RAW 264.7 macrophages, and the mechanism involved in the ethanol-induced TLR4 activation. Our results show that while ethanol, at high concentrations (100 mM) or in the presence of the LPS, suppresses the TLR4 response, low to moderate levels (10–50 mM) activate the TLR4 response and triggers the stimulation of the mitogen-activated protein kinases (MAPKs) and the transcription factor NF-κB pathways, leading to the production of nitric oxide (NO) and inflammatory cytokines. Pre-treatment with anti-TLR4 Abs abolishes the effects of ethanol on the production of cytokines. We also present evidence that stimulation with either ethanol or LPS induces translocation and clustering of TLR4 and signaling molecules (IRAK and MAPKs) into lipid rafts. Treatment with either streptolysin-O or saponin, lipid rafts disrupting agents, abolishes the ethanol-induced activation of the TLR4/IL-1RI signaling pathway. In summary, the present results demonstrate that low to moderate concentrations of ethanol are capable of stimulating TLR4/IL-1RI response, and provide evidence of a novel mechanism by which ethanol, through its interaction with membrane rafts, can promote TLR4/IL-1RI recruitment and signaling.

3.1059 Human Immunodeficiency Virus Type 1 Nef Recruits the Guanine Exchange Factor Vav1 via an Unexpected Interface into Plasma Membrane Microdomains for Association with p21-Activated Kinase 2 Activity
Rauch, S., Pulkkinen, K., Saksela, K. And Fackler, O.T.
J. Virol., ***82(6)***, *2918-2929 (2008)*

Alterations of T-cell receptor signaling by human immunodeficiency virus type 1 (HIV-1) Nef involve its association with a highly active subpopulation of p21-activated kinase 2 (PAK2) within a dynamic signalosome assembled in detergent-insoluble membrane microdomains. Nef-PAK2 complexes contain the GTPases Rac and Cdc42 as well as a factor providing guanine nucleotide exchange factor (GEF) activity for Rac/Cdc42. However, the identity of this GEF has remained controversial. Previous studies suggested the association of Nef with at least three independent GEFs, Vav, DOCK2/ELMO1, and βPix. Here we used a broad panel of approaches to address which of these GEFs is involved in the functional interaction of Nef with PAK2 activity. Biochemical fractionation and confocal microscopy revealed that Nef recruits Vav1, but not DOCK2/ELMO1 or βPix, to membrane microdomains. Transient RNAi knockdown, analysis of cell lines defective for expression of Vav1 or DOCK2 as well as use of a βPix binding-deficient PAK2 variant confirmed a role for Vav1 but not DOCK2 or βPix in Nef's association with PAK2 activity. Nef-mediated microdomain recruitment of Vav1 occurred independently of the Src homology 3 domain binding PxxP motif, which is known to connect Nef to many cellular signaling processes. Instead, a recently described protein interaction surface surrounding Nef residue F195 was identified as critical for Nef-mediated raft recruitment of Vav1. These results identify Vav1 as a relevant component of the Nef-PAK2 signalosome and provide a molecular basis for the role of F195 in formation of a catalytically active Nef-PAK2 complex.

3.1060 Signal sequence- and translation-independent mRNA localization to the endoplasmic reticulum
Pyhtila, B. et al
RNA, ***14***, *445-453 (2008)*

The process of mRNA localization typically utilizes *cis*-targeting elements and *trans*-recognition factors to direct the compartmental organization of translationally suppressed mRNAs. mRNA localization to the endoplasmic reticulum (ER), in contrast, occurs via a co-translational, signal sequence/signal recognition particle (SRP)-dependent mechanism. We have utilized cell fractionation/cDNA microarray analysis, shRNA-mediated suppression of SRP expression, and mRNA reporter construct studies to define the role of the SRP pathway in ER-directed mRNA localization. Cell fractionation studies of mRNA partitioning between the cytosol and ER demonstrated the expected enrichment of cytosolic/nucleoplasmic protein-encoding mRNAs and secretory/integral membrane protein-encoding mRNAs in the cytosol and ER fractions, respectively, and identified a subpopulation of cytosolic/nucleoplasmic protein-encoding mRNAs in the membrane-bound mRNA pool. The latter finding suggests a signal sequence-independent pathway of ER-directed mRNA localization. Extending from these findings, mRNA partitioning was examined in stable SRP54 shRNA knockdown HeLa cell lines. shRNA-directed reductions in SRP did not globally alter mRNA partitioning patterns, although defects in membrane protein processing were observed, further suggesting the existence of multiple pathways for mRNA localization to the ER. ER localization of GRP94-encoding mRNA was observed when translation was disabled by mutation of the start codon/insertion of a 5'UTR stem–loop structure or upon deletion of the encoded signal sequence. Combined, these data indicate that the mRNA localization to the ER can be conferred independent of the signal sequence/SRP pathway and suggest that mRNA localization to the ER may utilize *cis*-encoded targeting information.

3.1061 Myo2p, a class V myosin in budding yeast, associates with a large ribonucleic acid–protein complex that contains mRNAs and subunits of the RNA-processing body
Chang, W. et al
RNA, ***14***, *491-502 (2008)*

Myo2p is an essential class V myosin in budding yeast with several identified functions in organelle trafficking and spindle orientation. The present study demonstrates that Myo2p is a component of a large RNA-containing complex (Myo2p–RNP) that is distinct from polysomes based on sedimentation analysis and lack of ribosomal subunits in the Myo2p–RNP. Microarray analysis of RNAs that coimmunoprecipitate with Myo2p revealed the presence of a large number of mRNAs in this complex. The Myo2p–RNA complex is in part composed of the RNA processing body (P-body) based on coprecipitation with P-body protein subunits and partial colocalization of Myo2p with P-bodies. P-body disassembly is

delayed in the motor mutant, *myo2-66*, indicating that Myo2p may facilitate the release of mRNAs from the P-body.

3.1062 Protein quality control: the who's who, the where's and therapeutic escapes
Roth, J. et al
Histochem. Cell Biol., ***129***, *163-177 (2008)*

In cells the quality of newly synthesized proteins is monitored in regard to proper folding and correct assembly in the early secretory pathway, the cytosol and the nucleoplasm. Proteins recognized as non-native in the ER will be removed and degraded by a process termed ERAD. ERAD of aberrant proteins is accompanied by various changes of cellular organelles and results in protein folding diseases. This review focuses on how the immunocytochemical labeling and electron microscopic analyses have helped to disclose the in situ subcellular distribution pattern of some of the key machinery proteins of the cellular protein quality control, the organelle changes due to the presence of misfolded proteins, and the efficiency of synthetic chaperones to rescue disease-causing trafficking defects of aberrant proteins.

3.1063 Insulin Internalizes GLUT2 in the Enterocytes of Healthy but Not Insulin-Resistant Mice
Tobin, V. et al
Diabetes, ***57***, *555-562 (2008)*

OBJECTIVES—A physiological adaptation to a sugar-rich meal is achieved by increased sugar uptake to match dietary load, resulting from a rapid transient translocation of the fructose/glucose GLUT2 transporter to the brush border membrane (BBM) of enterocytes. The aim of this study was to define the contributors and physiological mechanisms controlling intestinal sugar absorption, focusing on the action of insulin and the contribution of GLUT2-mediated transport.
RESEARCH DESIGN AND METHODS—The studies were performed in the human enterocytic colon carcinoma TC7 subclone (Caco-2/TC7) cells and in vivo during hyperinsulinemic-euglycemic clamp experiments in conscious mice. Chronic high-fructose or high-fat diets were used to induce glucose intolerance and insulin resistance in mice.
RESULTS AND CONCLUSIONS—In Caco-2/TC7 cells, insulin action diminished the transepithelial transfer of sugar and reduced BBM and basolateral membrane (BLM) GLUT2 levels, demonstrating that insulin can target sugar absorption by controlling the membrane localization of GLUT2 in enterocytes. Similarly, in hyperinsulinemic-euglycemic clamp experiments in sensitive mice, insulin abolished GLUT2 (i.e., the cytochalasin B-sensitive component of fructose absorption), decreased BBM GLUT2, and concomitantly increased intracellular GLUT2. Acute insulin treatment before sugar intake prevented the insertion of GLUT2 into the BBM. Insulin resistance in mice provoked a loss of GLUT2 trafficking, and GLUT2 levels remained permanently high in the BBM and low in the BLM. We propose that, in addition to its peripheral effects, insulin inhibits intestinal sugar absorption to prevent excessive blood glucose excursion after a sugar meal. This protective mechanism is lost in the insulin-resistant state induced by high-fat or high-fructose feeding.

3.1064 Phospholipid actions on PGHS-1 and -2 cyclooxygenase kinetics
Doyen, J.R., Yucer, N., Lichtenberger, L.M. and Kulmacz, R.J.
Prostaglandins & Other Lipid Mediators, ***85(3-4)***, *134-143 (2008)*

Cyclooxygenase (COX) catalysis by prostaglandin H synthase (PGHS) is a key control step for regulation of prostanoid biosynthesis. Both PGHS isoforms are integral membrane proteins and their substrate fatty acids readily partition into membranes, but the impact of phospholipids and lipid membranes on COX catalysis and the actions of COX inhibitors are not well understood. We have characterized the COX kinetics and ibuprofen inhibition of the purified PGHS isoforms in the presence of phosphatidylcholine (PC) with varying acyl chain structure and physical state. PC was found to directly inhibit COX activity, with non-competitive inhibition by PC monomers binding away from the COX active site and competitive inhibition by micellar/bilayer forms of PC due to sequestration of the arachidonate substrate. Competitive inhibition by native membranes was observed in a comparison of COX kinetics in sheep seminal vesicle microsomes before and after solubilization of PGHS-1. PC liposomes significantly increase the inhibitory potency of ibuprofen against both PGHS isoforms without changing the reversible character of ibuprofen action or requiring binding of PGHS to the liposomes. These results suggest a useful conceptual framework for analyzing the complex interactions among the PGHS proteins, substrates, inhibitors and phospholipid.

3.1065 Multiple functions of ergosterol in the fission yeast Schizosaccharomyces pombe
Iwaki, T. et al
*Microbiology, **154**, 830-841 (2008)*

Sterols are a major class of membrane lipids in eukaryotes. In *Schizosaccharomyces pombe*, sterol 24-C-methyltransferase (Erg6p), C-8 sterol isomerase (Erg2p), C-5 sterol desaturase (Erg31p, Erg32p), C-22 sterol desaturase (Erg5p) and C-24 (28) sterol reductase (Sts1p/Erg4p) have been predicted, but not yet determined, to catalyse a sequence of reactions from zymosterol to ergosterol. Disruption mutants of these genes were unable to synthesize ergosterol, and most were tolerant to the polyene drugs amphotericin B and nystatin. Disruption of *erg31*$^{+}$ or *erg32*$^{+}$ did not cause ergosterol deficiency or tolerance to polyene drugs, indicating that the two C-5 sterol desaturases have overlapping functions. GFP-tagged DRM (detergent-resistant membrane)-associated protein Pma1p localized to the plasma membrane in *erg*Δ mutants. DRM fractionation revealed that the association between Pma1-GFP and DRM was weakened in *erg6*Δ but not in other *erg* mutants. Several GFP-tagged plasma membrane proteins were tested, and an amino acid permease homologue, SPBC359.03c, was found to mislocalize to intracellular punctate structures in the *erg*Δ mutants. These results indicate that these proteins are responsible for ergosterol biosynthesis in fission yeast, similar to the situation in *Saccharomyces cerevisiae*. Furthermore, in fission yeast, ergosterol is important for plasma membrane structure and function and for localization of plasma membrane proteins.

3.1066 CFTR in a lipid raft-TNFR1 complex modulates gap junctional intercellular communication and IL-8 secretion
Dudez, T. et al
*Eur. J. Lab. Invest., **38(Suppl.1-2)**, 20, abstract 54*

Background: Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause a chronic inflammatory response in the lung of patients with Cystic Fibrosis (CF). Defect in TNF-a signaling through the Src family tyrosine kinases (SFKs) has been reported in CF cells, and shown to unable the regulation of gap junctional communication (GJIC). Here, we sought to elucidate the mechanisms linking TNF-a signaling to the functions of CFTR at the molecular level. Materials and methods: Detergent-resistant membran microdomains
(DRMs), or lipid rafts, were isolated using the Optiprep method from MDCKI cells expressing wild-type (WtCFTR) or mutant CFTR lacking its PDZ-interacting motif (CFTR-aTRL), and from the human glandular Calu-3 cell line endogenously expressing CFTR. GJIC was evaluated by dye coupling and IL-8 secretion by dot blots.
Results: TNF-a increased the amount of Wt-CFTR but not CFTR-aTRL in DRMs. This recruitment was modulated by SFK activity and associated with DRM localization of TNFR1 and c-Src. Activation of TNFR1 signaling also decreased GJIC and markedly stimulated IL-8 production in WtCFTR cells. The absence of CFTR in DRMs was associated with abnormal TNFR1 signaling as revealed by no recruitment of TNFR1 and c-Src to lipid rafts in CFTR-aTRL cells and loss of regulation of GJIC and IL-8 secretion. We further show in Calu-3 cells that endogen CFTR is present at the cell surface in DRMs, which amount is enhanced by TNF-a and contributes to IL-8 secretion. Conclusions: Localization of CFTR to lipid rafts in association with c-Src and TNFR1 provides a responsive signaling complex to regulate GJIC and cytokine signaling. Disruption of
CFTR interactions with component of this complex may lead to abnormalities relevant for the CF pathogenesis.

3.1067 A Lipid-mediated Quality Control Process in the Golgi Apparatus in Yeast
Pineau, L. et al
*Mol. Biol. Cell, **9**, 807-821 (2008)*

When heme biosynthesis is disrupted, the yeast *Saccharomyces cerevisiae* becomes unable to synthesize its major sterol, ergosterol, and desaturate fatty acids. We took advantage of this physiological peculiarity to evaluate the consequences of ergosterol and/or unsaturated fatty acid (UFA) depletions on the biogenesis of a model polytopic plasma membrane protein, the uracil permease Fur4p. We show that under UFA shortage, which results in low amounts of diunsaturated phospholipid species, and under ergosterol depletion, Fur4p is prematurely routed from the Golgi apparatus to the vacuolar lumen in a process that requires the ubiquitin ligase Rsp5p. Interestingly, this diversion is not correlated to Fur4p exclusion from detergent-resistant membranes. In an independent set of experiments, we show that Fur4p targeting to the plasma membrane depends on phosphatidylethanolamine amounts and more specifically on the propensity

of this phospholipid to form a hexagonal phase. In light of recent literature, we propose a model in which ergosterol and diunsaturated phospholipid species maintain optimal membrane curvature for Fur4p to evade the Golgi quality control process and to be properly delivered to its normal destination.

3.1068 Phospholipid and glycolipid composition of acidocalcisomes of Trypanosoma cruzi
Salto, M.L., Kuhlenschmidt, T., Kuhlenschmidt, M., de Lederkremer, R.M. and Docampo, R.
*Mol. Biochem. Parasitol., **158**, 120-130 (2008)*

Highly purified acidocalcisomes from *Trypanosoma cruzi* epimastigotes were obtained by differential centrifugation and iodixanol gradient ultracentrifugation. Lipid analysis of acidocalcisomes revealed the presence of low amounts of 3β-hydroxysterols and predominance of phospholipids. Alkylacyl phosphatidylinositol (16:0/18:2), diacyl phosphatidylinositol (18:0/18:2), diacyl phosphatidylcholine (16:0/18:2; 16:1/18:2; 16:2/18:2; 18:1/18:2 and 18:2/18:2), and diacyl phosphatidylethanolamine (16:0/18:2 and 16:1/18:2) were the only phospholipids characterized by electrospray ionization-mass spectrometry (ESI-MS). Incubation of epimastigotes with [^{3}H]-mannose and isolation of acidocalcisomes allowed the detection of a glycoinositolphospholipid (GIPL) in these organelles. The sugar content of the acidocalcisomal GIPL was similar to that of the GIPL present in a microsomal fraction but the amount of galactofuranose and inositol with respect to the other monosaccharides was lower, suggesting a different chemical structure. Taken together, these results indicate that acidocalcisomes of *T. cruzi* have a distinct lipid and carbohydrate composition.

3.1069 FcγRI (CD64) resides constitutively in lipid rafts
Beekman, J.M., van der Linden, J.A., van de Winkel, J.G.J. and Leusen, J.H.W.
*Immunol. Lett., **116**, 149-155 (2008)*

Cellular membranes contain microdomains known as 'lipid rafts' or detergent-insoluble microdomains (DRM), enriched in cholesterol and sphingolipids. DRM can play an important role in many cellular processes, including signal transduction, cytoskeletal organization, and pathogen entry. Many receptors like T cell receptors, B cell receptors and IgE receptors have been shown to reside in DRM. The majority of these receptors depend on multivalent ligand interaction to associate with these microdomains. We, here, study association between the high affinity IgG receptor, FcγRI (CD64), and membrane microdomains. FcγRI is a 72 kDa type I glycoprotein that can mediate phagocytosis of opsonized pathogens, but can also effectively capture small immune complexes, and facilitates antigen presentation. We found FcγRI to predominantly reside within detergent-insoluble buoyant membranes, together with FcRγ-chain, but independent of cross-linking ligand. With the use of confocal imaging, FcγRI was found to co-patch with GM1, a microdomain-enriched glycolipid. Depletion of cellular cholesterol, furthermore, modulated FcγRI–ligand interactions. These data indicated FcγRI to reside within lipid rafts without prior triggering of the receptor.

3.1070 Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria
Vande Velde, C., Miller, T.M., Cashman, N.R. and Cleveland, D.W.
*PNAS, **105(10)**, 4022-4027 (2008)*

Mutations in copper/zinc superoxide dismutase (SOD1) are causative for dominantly inherited amyotrophic lateral sclerosis (ALS). Despite high variability in biochemical properties among the disease-causing mutants, a proportion of both dismutase-active and -inactive mutants are stably bound to spinal cord mitochondria. This mitochondrial proportion floats with mitochondria rather than sedimenting to the much higher density of protein, thus eliminating coincidental cosedimentation of protein aggregates with mitochondria. Half of dismutase-active and ≈90% of dismutase-inactive mutant SOD1 is bound to mitochondrial membranes in an alkali- and salt-resistant manner. Sensitivity to proteolysis and immunoprecipitation with an antibody specific for misfolded SOD1 demonstrate that in all mutant SOD1 models, misfolded SOD1 is deposited onto the cytoplasmic face of the outer mitochondrial membrane, increasing antigenic accessibility of the normally structured electrostatic loop. Misfolded mutant SOD1 binding is both restricted to spinal cord and selective for mitochondrial membranes, implicating exposure to mitochondria of a misfolded mutant SOD1 conformer mediated by a unique, tissue-selective composition of cytoplasmic chaperones, components unique to the cytoplasmic face of spinal mitochondria to which misfolded SOD1 binds, or misfolded SOD1 conformers unique to spinal cord that have a selective affinity for mitochondrial membranes.

3.1071 **Carboxypeptidase M and Kinin B1 Receptors Interact to Facilitate Efficient B1 Signaling from B2 Agonists**
Zhang, X., Tan, F., Zhang, Y. And Skidgel, R.
J. Biol. Chem., ***283(12)***, *7994-8004 (2008)*

Kinin B1 receptor (B1R) expression is induced by injury or inflammatory mediators, and its signaling produces both beneficial and deleterious effects. Kinins cleaved from kininogen are agonists of the B2R and must be processed by a carboxypeptidase to generate B1R agonists des-Arg^{9}-bradykinin or des-Arg^{10}-kallidin. Carboxypeptidase M (CPM) is a membrane protein potentially well suited for this function. Here we show that CPM expression is required to generate a B1R-dependent increase in $[Ca^{2+}]_i$ in cells stimulated with B2R agonists kallidin or bradykinin. CPM and the B1R interact on the cell membrane, as shown by co-immunoprecipitation, cross-linking, and fluorescence resonance energy transfer analysis. CPM and B1R are also co-localized in lipid raft/caveolin-enriched membrane fractions, as determined by gradient centrifugation. Treatment of cells co-expressing CPM and B1R with methyl-β-cyclodextrin to disrupt lipid rafts reduced the B1R-dependent increase in $[Ca^{2+}]_i$ in response to B2R agonists, whereas cholesterol treatment enhanced the response. A monoclonal antibody to the C-terminal β-sheet domain of CPM reduced the B1R response to B2R agonists without inhibiting CPM. Cells expressing a novel fusion protein containing CPM at the N terminus of the B1R also increased $[Ca^{2+}]_i$ when stimulated with B2R agonists, but the response was not reduced by methyl-β-cyclodextrin or CPM antibody. A B1R- and CPM-dependent calcium signal in response to B2R agonist bradykinin was also found in endothelial cells that express both proteins. Thus, a close relationship of B1Rs and CPM on the membrane is required for efficiently generating B1R signals, which play important roles in inflammation.

3.1072 **Over-expression of mammalian sialidase NEU3 reduces Newcastle disease virus entry and propagation in COS7 cells**
Anastasia, L. et al
Biochim. Biophys. Acta, ***1780(3)***, *504-512 (2008)*

The paramyxovirus Newcastle Disease Virus (NDV) binds to sialic acid-containing glycoconjugates, sialoglycoproteins and sialoglycolipids (gangliosides) of host cell plasma membrane through its hemagglutinin-neuraminidase (sialidase) HN glycoprotein. We hypothesized that the modifications of the cell surface ganglioside pattern determined by over-expression of the mammalian plasma-membrane associated, ganglioside specific, sialidase NEU3 would affect the virus-host cell interactions. Using COS7 cells as a model system, we observed that over-expression of the murine MmNEU3 did not affect NDV binding but caused a marked reduction in NDV infection and virus propagation through cell–cell fusion. Moreover, since GD1a was greatly reduced in COS7 cells following NEU3-over-expression, we added [^{3}H]-labelled GD1a to COS7 cells under conditions that block intralysosomal metabolic processing, and we observed a marked increase of GD1a cleavage to GM1 during NDV infection, indicating a direct involvement of the virus sialidase and host cell GD1a in NDV infectivity. Therefore, the decrease of GD1a in COS7 cell membrane upon MmNEU3 over-expression is likely to be instrumental to NDV reduced infection. Evidence was also provided for the preferential association of NDV-HN at 4 °C to detergent resistant microdomains (DRMs) of COS7 cells plasma membranes.

3.1073 **Fractionation of Subcellular Membrane Vesicles of Epithelial and Nonepithelial Cells by OptiPrep™ Density Gradient Ultracentrifugation**
Li, X. and Donowitz, M.
Methods Mol. Biol., ***440***, *97-110 (2008)*

Density gradient ultracentrifugation (DGUC) is widely used for physical isolation (enrichment rather than purification) of subcellular membrane vesicles. It has been a valuable tool to study specific subcellular localization and dynamic trafficking of proteins. While sucrose has been the main component of density gradients, a few years ago synthetic OptiPrep™ (iodixanol) began being used for separation of organelles because of its iso-osmotic property. Here, we describe a detailed protocol for density gradient fractionation of various mammalian subcellular vesicles, including endoplasmic reticulum (ER), Golgi apparatus, endosomes, and lipid rafts, as well as apical and basolateral membranes of polarized epithelial cells.

3.1074 **Determination of Genuine Residents of Plant Endomembrane Organelles using Isotope Tagging and Multivariate Statistics**
Lilley, K.S. and Dunkley, T.P.J.
Methods Mol. Biol., ***432***, *373-387 (2008)*

The knowledge of the localization of proteins to a particular subcellular structure or organelle is an important step towards assigning function to proteins predicted by genome-sequencing projects that have yet to be characterized. Moreover, the localization of novel proteins to organelles also enhances our understanding of the functions of organelles. Many organelles cannot be purified. In several cases where the degree of contamination by organelles with similar physical parameters to the organelle being studied has gone unchecked, this has lead to the mis-localization of proteins. Recently, several techniques have emerged, which depend on characterization of the distribution pattern of organelles partially separated using density centrifugation by quantitative proteomics approaches. Here, we discuss one of these approaches, the localization of organelle proteins by isotope tagging (LOPIT) where the distribution patterns of organelles are assessed by measuring the relative abundance of proteins between fractions along the length of density gradients using stable isotope-coded tags. The subcellular localizations of proteins can be determined by comparing their distributions to those of previously localized proteins by assuming that proteins that belong to the same organelle will cofractionate in density gradients. Analysis of distribution patterns can be achieved by employing multivariate statistical methods such as principal component analysis and partial least squares discriminate analysis. In this chapter, we focus on the use of the LOPIT technique in the assignment of membrane proteins to the plant Golgi apparatus and endoplasmic reticulum.

3.1075 Quantitative Proteomic Analysis to Profile Dynamic Changes in the Spatial Distribution of Cellular Proteins

Yan, W., Hwang, D. and Aebersold, R.
Methods Mol. Biol., ***432****, 389-401 (2008)*

Organelle protein profiles have traditionally been analyzed by subcellular fractionation of a specific organelle followed by the identification of the protein components of specific fractions containing the target organelle(s) using mass spectrometry (MS). However, because of limited resolution of the available fractionation methods, it is often difficult to isolate and thus profile pure organelles. Furthermore, many proteins (e.g., secretory proteins) are often observed to dynamically shuttle between organelles. Therefore, the determination of their true cellular localization requires the concurrent analysis of multiple organelles from the same cell lysate. Here, we report an integrated experimental approach that simultaneously profiles multiple organelles. It is based on the subcellular fractionation of cell lysates by density gradient centrifugation, iTRAQ labeling, and MS analysis of the proteins in selected fractions and principal component analysis (PCA) of the resulting quantitative proteomic data. Quantitative signature patterns of several organelles, including the ribosome, mitochondria, proteasome, lysosome, endoplasmic reticulum (ER), and Golgi apparatus, have been acquired from a single multiplexed proteomic assay using iTRAQ reagents. Through comparison PCA, we compare organelle profiles from cells under different physiological conditions to investigate changes in organelle profiles between control and perturbed samples. Such quantitative proteomics-based subcellular profiling methods thus provide useful tools to dissect the organization of cellular proteins into functional units and to detect dynamic changes in their protein composition.

3.1076 Free Flow Isoelectric Focusing
A Method for the Separation of Both Hydrophilic and Hydrophobic Proteins of Rat Liver Peroxisomes

Islinger, M. and Weber, G.
Methods Mol. Biol., ***432****, 199-215 (2008)*

Peroxisomes take part in various metabolic pathways related to the regulation of lipid homeostasis. Although detailed information on the enzymes involved in the peroxisomal lipid metabolism was acquired in the past, the mechanisms of metabolic exchange between peroxisomes and the cytosol or other organelles still remain an enigma. Therefore, a detailed analysis of the peroxisomal membrane proteome could help identify potential metabolite transporters. However, because of their highly hydrophobic character, membrane proteins tend to precipitate in aqueous media, making their fractionation still a challenging task. To overcome these obstacles, we have elaborated a protocol for the separation of both hydrophilic as well as hydrophobic proteins using free flow isoelectric focusing (FF-IEF). Similar to traditional gel-based isoelectric focusing, a denaturing electrophoresis buffer containing a mixture of urea, thiourea and detergents is applied to keep highly hydrophobic proteins in solution. Electrophoresis is conducted on a BD Free Flow Electrophoresis System with a linear pH gradient from 3 to 10 and sampled into 96 fractions. As a second dimension, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) is used to further separate and visualize the protein pattern of the peroxisomal subfractions

of matrix, peripheral and integral membrane proteins. The identification of the known peroxisomal membrane proteins PMP22, PMP70 as well as mGST in the subsequent matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of the 100 most prominent protein bands has documented the suitability of this new technique for the analysis of hydrophobic proteins.

3.1077 Sequencing and analysis of the gene-rich space of cowpea
Timko, M.P. et al
*BMC Genomics, **9**, 103-122 (2008)*

Background
Cowpea, *Vigna unguiculata* (L.) Walp., is one of the most important food and forage legumes in the semi-arid tropics because of its drought tolerance and ability to grow on poor quality soils. Approximately 80% of cowpea production takes place in the dry savannahs of tropical West and Central Africa, mostly by poor subsistence farmers. Despite its economic and social importance in the developing world, cowpea remains to a large extent an underexploited crop. Among the major goals of cowpea breeding and improvement programs is the stacking of desirable agronomic traits, such as disease and pest resistance and response to abiotic stresses. Implementation of marker-assisted selection and breeding programs is severely limited by a paucity of trait-linked markers and a general lack of information on gene structure and organization. With a nuclear genome size estimated at ~620 Mb, the cowpea genome is an ideal target for reduced representation sequencing.
Results
We report here the sequencing and analysis of the gene-rich, hypomethylated portion of the cowpea genome selectively cloned by methylation filtration (MF) technology. Over 250,000 gene-space sequence reads (GSRs) with an average length of 610 bp were generated, yielding ~160 Mb of sequence information. The GSRs were assembled, annotated by BLAST homology searches of four public protein annotation databases and four plant proteomes (*A. thaliana*, *M. truncatula*, *O. sativa*, and *P. trichocarpa*), and analyzed using various domain and gene modeling tools. A total of 41,260 GSR assemblies and singletons were annotated, of which 19,786 have unique GenBank accession numbers. Within the GSR dataset, 29% of the sequences were annotated using the Arabidopsis Gene Ontology (GO) with the largest categories of assigned function being catalytic activity and metabolic processes, groups that include the majority of cellular enzymes and components of amino acid, carbohydrate and lipid metabolism. A total of 5,888 GSRs had homology to genes encoding transcription factors (TFs) and transcription associated factors (TAFs) representing about 5% of the total annotated sequences in the dataset. Sixty-two (62) of the 64 well-characterized plant transcription factor (TF) gene families are represented in the cowpea GSRs, and these families are of similar size and phylogenetic organization to those characterized in other plants. The cowpea GSRs also provides a rich source of genes involved in photoperiodic control, symbiosis, and defense-related responses. Comparisons to available databases revealed that about 74% of cowpea ESTs and 70% of all legume ESTs were represented in the GSR dataset. As approximately 12% of all GSRs contain an identifiable simple-sequence repeat, the dataset is a powerful resource for the design of microsatellite markers.
Conclusion
The availability of extensive publicly available genomic data for cowpea, a non-model legume with significant importance in the developing world, represents a significant step forward in legume research. Not only does the gene space sequence enable the detailed analysis of gene structure, gene family organization and phylogenetic relationships within cowpea, but it also facilitates the characterization of syntenic relationships with other cultivated and model legumes, and will contribute to determining patterns of chromosomal evolution in the Leguminosae. The micro and macrosyntenic relationships detected between cowpea and other cultivated and model legumes should simplify the identification of informative markers for marker-assisted trait selection and map-based gene isolation necessary for cowpea improvement.

3.1078 Endosomal NADPH oxidase regulates c-Src activation following hypoxia/reoxygenation injury
Li, Q., Zhang, Y., Marden, J.J., Banf, B. And Engelhardt, J.F.
*Biochem. J., **411**, 531-541 (2008)*

c-Src has been shown to activate NF-κB (nuclear factor κB) following H/R (hypoxia/reoxygenation) by acting as a redox-dependent IκBα (inhibitory κB) tyrosine kinase. In the present study, we have investigated the redox-dependent mechanism of c-Src activation following H/R injury and found that ROS (reactive oxygen species) generated by endosomal Noxs (NADPH oxidases) are critical for this process. Endocytosis following H/R was required for the activation of endosomal Noxs, c-Src activation, and the ability of c-Src to tyrosine-phosphorylate IκBα. Quenching intra-endosomal ROS during reoxygenation inhibited c-Src activation without affecting c-Src recruitment from the plasma membrane to endosomes. However, siRNA (small interfering RNA)-mediated knockdown of Rac1 prevented c-Src recruitment into the endosomal compartment following H/R. Given that Rac1 is a known activator of Nox1 and Nox2, we investigated whether these two proteins were required for c-Src activation in Nox-deficient primary fibroblasts. Findings from these studies suggest that both Nox1 and Nox2 participate in the initial redox activation of c-Src following H/R. In summary, our results suggest that Rac1-dependent Noxs play a

critical role in activating c-Src following H/R injury. This signalling pathway may be a useful therapeutic target for ischaemia/reperfusion-related diseases.

3.1079 Proteomic Analysis of Highly Purified Peroxisomes from Etiolated Soybean Cotyledons
Arai, Y., Hayashi, M. and Nishimura, M.
Plant Cell. Physiol., ***49(4)***, *526-539 (2008)*

To identify previously unknown peroxisomal proteins, we established an optimized method for isolating highly purified peroxisomes from etiolated soybean cotyledons using Percoll density gradient centrifugation followed by **iodixanol** density gradient centrifugation. Proteins in highly purified peroxisomes were separated by two-dimensional PAGE. We performed peptide mass fingerprinting of proteins separated in the gel with matrix-assisted laser desorption ionization time-of-flight mass spectrometry and used the peptide mass fingerprints to search a non-redundant soybean expressed sequence tag database. We succeeded in assigning 92 proteins to 70 sequences in the database. Among them, proteins encoded by 30 sequences were judged to be located in peroxisomes. These included enzymes for fatty acid **β**-oxidation, the glyoxylate cycle, photorespiratory glycolate metabolism, stress response and metabolite transport. We also show experimental evidence that plant peroxisomes contain a short-chain dehydrogenase/reductase family protein, enoyl-CoA hydratase/isomerase family protein, 3-hydroxyacyl-CoA dehydrogenase-like protein and a voltage-dependent anion-selective channel protein.

3.1080 A fluorescent sphingolipid binding domain peptide probe interacts with sphingolipids and cholesterol-dependent raft domains
Hebbar, S. et al
J. Lipid Res., ***49***, *1077-1089 (2008)*

We have designed a tagged probe [sphingolipid binding domain (SBD)] to facilitate the tracking of intracellular movements of sphingolipids in living neuronal cells. SBD is a small peptide consisting of the SBD of the amyloid precursor protein. It can be conjugated to a fluorophore of choice and exogenously applied to cells, thus allowing for in vivo imaging. Here, we present evidence to describe the characteristics of the SBD association with the plasma membrane. Our experiments demonstrate that SBD binds to isolated raft fractions from human neuroblastomas and insect neuronal cells. In protein-lipid overlay experiments, SBD interacts with a subset of glycosphingolipids and sphingomyelin, consistent with its raft association in neurons. We also provide evidence that SBD is taken up by neuronal cells in a cholesterol- and sphingolipid-dependent manner via detergent-resistant microdomains. Furthermore, using fluorescence correlation spectroscopy to assay the mobility of SBD in live cells, we show that SBD's behavior at the plasma membrane is similar to that of the previously described raft marker cholera toxin B, displaying both a fast and a slow component. Our data suggest that fluorescently tagged SBD can be used to investigate the dynamic nature of glycosphingolipid-rich detergent-resistant microdomains that are cholesterol-dependent.

3.1081 CFTR in a lipid raft-TNFR1 complex modulates gap junctional intercellular communication and IL-8 secretion
Dudez, T. et al
Biochim. Biophys. Acta, ***1783***, *779-788 (2008)*

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause a chronic inflammatory response in the lung of patients with Cystic Fibrosis (CF). We have showed that TNF-∗ signaling through the Src family tyrosine kinases (SFKs) was defective as determined by an inability of TNF-∗ to regulate gap junctional communication (GJIC) in CF cells. Here, we sought to elucidate the mechanisms linking TNF-∗ signaling to the functions of CFTR at the molecular level. In a MDCKI epithelial cell model expressing wild-type (WtCFTR) or mutant CFTR lacking its PDZ-interacting motif (CFTR-ΔTRL), TNF-∗ increased the amount of WtCFTR but not CFTR-ΔTRL in detergent-resistant membrane microdomains (DRMs). This recruitment was modulated by SFK activity and associated with DRM localization of TNFR1 and c-Src. Activation of TNFR1 signaling also decreased GJIC and markedly stimulated IL-8 production in WtCFTR cells. In contrast, the absence of CFTR in DRMs was associated with abnormal TNFR1 signaling as revealed by no recruitment of TNFR1 and c-Src to lipid rafts in CFTR-ΔTRL cells and loss of regulation of GJIC and IL-8 secretion. These results suggest that localization of CFTR in lipid rafts in association with c-Src and TNFR1 provides a responsive signaling complex to regulate GJIC and cytokine signaling.

3.1082 Differential membrane compartmentalization of Ret by PTB-adaptor engagement
Lundgren, T.K., Luebke, M., Stenqvist, A. and Ernfors, P.
*FEBS J., **275**, 2055-2066 (2008)*

Glial cell line-derived neurotrophic factor family ligands act through the receptor tyrosine kinase Ret, which plays important roles during embryonic development for cell differentiation, survival, and migration. Ret signaling is markedly affected by compartmentalization of receptor complexes into membrane subdomains. Ret can propagate biochemical signaling from within concentrates in cholesterol-rich membrane microdomains or lipid rafts, or outside such regions, but the mechanisms for, and consequences of, Ret translocation between these membrane compartments remain largely unclear. Here we investigate the interaction of Shc and Frs2 phosphotyrosine-binding domain-containing adaptor molecules with Ret and their function in redistributing Ret to specialized membrane compartments. We found that engagement of Ret with the Frs2 adaptor results in an enrichment of Ret in lipid rafts and that signal transduction pathways and chemotaxis responses depend on the integrity of such rafts. The competing Shc adaptor did not promote Ret translocation to equivalent domains, and Shc-mediated effects were less affected by disruption of lipid rafts. However, by expressing a chimeric Shc protein that localizes to lipid rafts, we showed that biochemical signaling downstream of Ret resembled that of Ret signaling via Frs2. We have identified a previously unknown mechanism in which phosphotyrosine-binding domain-containing adaptors, by means of relocating Ret receptor complexes to lipid rafts, segregate diverse signaling and cellular functions mediated by Ret. These results reveal the existence of a novel mechanism that could, by subcellular relocation of Ret, work to amplify ligand gradients during chemotaxis.

3.1083 Sequestration of NF-κB Signaling Complexes in Lipid Rafts Contributes to Repression of NF-κB in T Lymphocytes under Hyperthermia Stress
Yan, G., Huang, J., Ruth Jarbadan, N., Jiang, Y. And Cheng, H.
*J. Biol. Chem., **283(18)**, 12489-12500 (2008)*

Sepsis causes extensive apoptosis of lymphocytes, a pathological condition that is frequently associated with hyperthermia. Heat stress has been implicated to repress the activation of an inflammatory mediator, nuclear factor of κB (NF-κB), which sensitizes cells to apoptosis mediated by inflammatory cytokine, tumor necrosis factor α. However, the molecular mechanism of hyperthermia-associated loss of T cells remains unclear. We show that hyperthermia causes rapid translocation of IκB kinase (IKK) and NF-κB complexes into the plasma membrane-associated lipid rafts in T cells. Heat stress induces aggregation of Carma1 in lipid rafts, which in turn recruits protein kinase Cθ (PKCθ) and Bcl10 to the microdomains, causing subsequent membrane translocation of the IKK and NF-κB signalosomes. Depletion of Carma1 and inhibition of PKCθ impair accumulation of NF-κB complexes in lipid rafts. Heat stress prohibits IκB kinase activity by sequestrating the IKK and NF-κB complexes in lipid rafts and by segregating the chaperone protein Hsp90, an essential cofactor for IKK, from the IKK complex. This process ultimately results in functional deficiency of NF-κB and renders T cells resistant to tumor necrosis factor α-induced activation of IKK, thereby contributing to the apoptotic loss of T lymphocytes in sepsis-associated hyperthermia.

3.1084 MALS-3 regulates polarity and early neurogenesis in the developing cerebral cortex
Srinivasan, K. et al
*Development, **135**, 1781-1790 (2008)*

Apicobasal polarity plays an important role in regulating asymmetric cell divisions by neural progenitor cells (NPCs) in invertebrates, but the role of polarity in mammalian NPCs is poorly understood. Here, we characterize the function of the PDZ domain protein MALS-3 in the developing cerebral cortex. We find that MALS-3 is localized to the apical domain of NPCs. Mice lacking all three MALS genes fail to localize the polarity proteins PATJ and PALS1 apically in NPCs, whereas the formation and maintenance of adherens junctions appears normal. In the absence of MALS proteins, early NPCs progressed more slowly through the cell cycle, and their daughter cells were more likely to exit the cell cycle and differentiate into neurons. Interestingly, these effects were transient; NPCs recovered normal cell cycle properties during late neurogenesis. Experiments in which MALS-3 was targeted to the entire membrane resulted in a breakdown of apicobasal polarity, loss of adherens junctions, and a slowing of the cell cycle. Our results suggest that MALS-3 plays a role in maintaining apicobasal polarity and is required for normal neurogenesis in the developing cortex.

3.1085 **Down-regulation of insulin-degrading enzyme by presenilin 1 V97L mutant potentially underlies increased levels of amyloid beta 42**
Qin, W. and Jia, J.
Eur. J. Neurosci., ***27***, *2425-2432 (2008)*

Amyloid beta (Aβ)42 plays a pivotal role in Alzheimer's disease. We previously reported a novel presenilin (PS)1 mutant (V97L) that was expressed in related patients with early onset Alzheimer's disease. We found that patients with the V97L mutation had increased levels of extracellular and intracellular Aβ42. Here we found that the increased extracellular level of Aβ42 was always accompanied by a reduction of insulin-degrading enzyme (IDE) activity on the plasma membranes. However, increase of intracellular Aβ42 was associated with decreased expression and activity of IDE in the cytosol and endoplasmic reticulum in the PS1 V97L mutant-transfected human SH-SY5Y cell line. These studies indicate that pathological levels of Aβ42 may be caused by the negative effects of PS1 (V97L) on IDE expression and activity. Our findings provide evidence for the molecular basis of familial Alzheimer's disease pathogenesis.

3.1086 **Aberrant Folding of Pathogenic Parkin Mutants: AGGREGATION VERSUS DEGRADATION**
Schlehe, J.S. et al
J. Biol. Chem., ***283(20)***, *13771-13779 (2008)*

Loss-of-function mutations in the Parkin gene (*PARK2*) are responsible for the majority of autosomal recessive Parkinson disease. A growing body of evidence indicates that misfolding and aggregation of Parkin is a major mechanism of Parkin inactivation, accounting for the loss-of-function phenotype of various pathogenic Parkin mutants. Remarkably, wild-type Parkin is also prone to misfolding under certain cellular conditions, suggesting a more general role of Parkin in the pathogenesis of Parkinson disease. We now show that misfolding of Parkin can lead to two phenotypes: the formation of detergent-insoluble, aggregated Parkin, or destabilization of *J. Neurochem.*, *105*Parkin resulting in an accelerated proteasomal degradation. By combining two pathogenic Parkin mutations, we could demonstrate that destabilization of Parkin is dominant over the formation of detergent-insoluble Parkin aggregates. Furthermore, a comparative analysis with HHARI, an E3 ubiquitin ligase with an RBR domain highly homologous to that of Parkin, revealed that folding of Parkin is specifically dependent on the integrity of the C-terminal domain, but not on the presence of a putative PDZ-binding motif at the extreme C terminus.

3.1087 **Activated Nuclear Metabotropic Glutamate Receptor mGlu5 Couples to Nuclear Gq/11 Proteins to Generate Inositol 1,4,5-Trisphosphate-mediated Nuclear Ca2+ Release**
Kumar, V., Jong, Y-J.I. and O'Malley, K.L.
J. Biol. Chem., ***283(20)***, *14072-14083 (2008)*

Recently we have shown that the metabotropic glutamate 5 (mGlu5) receptor can be expressed on nuclear membranes of heterologous cells or endogenously on striatal neurons where it can mediate nuclear Ca^{2+} changes. Here, pharmacological, optical, and genetic techniques were used to show that upon activation, nuclear mGlu5 receptors generate nuclear inositol 1,4,5-trisphosphate (IP_3) *in situ*. Specifically, expression of an mGlu5 F767S mutant in HEK293 cells that blocks $G_{q/11}$ coupling or introduction of a dominant negative $G\alpha_q$ construct in striatal neurons prevented nuclear Ca^{2+} changes following receptor activation. These data indicate that nuclear mGlu5 receptors couple to $G_{q/11}$ to mobilize nuclear Ca^{2+}. Nuclear mGlu5-mediated Ca^{2+} responses could also be blocked by the phospholipase C (PLC) inhibitor, U73122 [GenBank] , the phosphatidylinositol (PI) PLC inhibitor 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (ET-18-OCH_3), or by using small interfering RNA targeted against PLCβ1 demonstrating that PI-PLC is involved. Direct assessment of inositol phosphate production using a PIP2/IP_3 "biosensor" revealed for the first time that IP_3 can be generated in the nucleus following activation of nuclear mGlu5 receptors. Finally, both IP_3 and ryanodine receptor blockers prevented nuclear mGlu5-mediated increases in intranuclear Ca^{2+}. Collectively, this study shows that like plasma membrane receptors, activated nuclear mGlu5 receptors couple to $G_{q/11}$ and PLC to generate IP_3-mediated release of Ca^{2+} from Ca^{2+}-release channels in the nucleus. Thus the nucleus can function as an autonomous organelle independent of signals originating in the cytoplasm, and nuclear mGlu5 receptors play a dynamic role in mobilizing Ca^{2+} in a specific, localized fashion.

3.1088 **Phorbol ester induced trafficking-independent regulation and enhanced phosphorylation of the dopamine transporter associated with membrane rafts and cholesterol**
Foster, J.D., Adkins, S.D., Lever, J.R. and Vaughan, R.A.

J. Neurochem., ***105***, *1683-1699 (2008)*

We examined the mechanisms involved in protein kinase C (PKC)-dependent down-regulation of dopamine transporter (DAT) activity and cell surface expression by treating heterologously expressing cells with the clathrin-mediated endocytosis inhibitor concanavalin A (Con A) or the cholesterol depleter/membrane raft disrupter methyl-β-cyclodextrin (MβC) prior to treatment with the PKC activator phorbol 12-myristate, 13-acetate (PMA). Con A blocked PMA-induced surface reductions of DAT but only partially inhibited down-regulation, while MβC partially blocked down-regulation but did not inhibit loss of cell surface DAT, demonstrating that PKC-induced DAT down-regulation occurs by a combination of trafficking and non-trafficking processes. Using density-gradient centrifugation, we found that DATs are distributed approximately equally between Triton-insoluble, cholesterol-rich membrane rafts and Triton-soluble non-raft membranes. DATs in both populations are present at the cell surface and are active for dopamine and cocaine binding. PMA-induced loss of cell surface DAT occurred only from non-raft populations, demonstrating that non-raft DATs are regulated by trafficking events and indicating the likelihood that the cholesterol-dependent non-trafficking regulatory mechanism occurs in rafts. PMA did not affect the DAT raft-non-raft distribution but stimulated the phosphorylation of DAT to a substantially greater level in rafts than non-rafts. These findings reveal a previously unknown role for cholesterol in DAT function and demonstrate the presence of distinct subcellular DAT populations that possess multiple regulatory differences that may impact dopaminergic neurotransmission.

3.1089 Epstein-Barr Virus Latent Membrane Protein 1 Induces Expression of the Epidermal Growth Factor Receptor through Effects on Bcl-3 and STAT3
Kung,, C-P. and Raab-Traub, N.
J. Virol., ***82(11)***, *5486-5493 (2008)*

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) activates multiple signaling pathways. Two regions, C-terminal-activating region 1 (CTAR1) and CTAR2, have been identified within the cytoplasmic carboxy terminal domain that activates NF-κB. CTAR2 activates the canonical NF-κB pathway, which includes p50/p65 complexes. CTAR1 can activate both the canonical and noncanonical pathways to produce multiple distinct NF-κB dimers, including p52/p50, p52/p65, and p50/p50. CTAR1 also uniquely upregulates the epidermal growth factor receptor (EGFR) in epithelial cells. Increased p50-Bcl-3 complexes have been detected by chromatin precipitation on the NF-κB consensus motifs within the *egfr* promoter in CTAR1-expressing epithelial cells and nasopharyngeal carcinoma cells. In this study, the mechanism responsible for the increase in Bcl-3 has been further investigated. The data indicate that LMP1-CTAR1 induces Bcl-3 mRNA and increases the nuclear translocation of both Bcl-3 and p50. LMP1-CTAR1 constitutively activates STAT3, and this activation was not due to the induction of interleukin 6 (IL-6). In LMP1-CTAR1-expressing cells, increased levels of activated STAT3 were detected by chromatin immunoprecipitation on STAT-binding sites located within both the promoter and the second intron of Bcl-3. A STAT3 inhibitor significantly reduced the activation of STAT3, as well as the CTAR1-mediated upregulation of Bcl-3 and EGFR. These data suggest that LMP1 activates distinct forms of NF-κB through multiple pathways. In addition to activating the canonical and noncanonical pathways, LMP1-CTAR1 constitutively activates STAT3 and increases Bcl-3. The increased nuclear Bcl-3 and p50 homodimer complexes positively regulate EGFR expression. These results indicate that LMP1 likely regulates distinct cellular genes by activating specific NF-κB pathways.

3.1090 Involvement of human CD44 during Cryptococcus neoformans infection of brain microvascular endothelial cells
Jong, A. et al
Cellular. Microbiol., ***10(6)***, *1313-1326 (2008)*

Pathogenic yeast *Cryptococcus neoformans* causes devastating cryptococcal meningoencephalitis. Our previous studies demonstrated that *C. neoformans* hyaluronic acid was required for invasion into human brain microvascular endothelial cells (HBMEC), which constitute the blood–brain barrier. In this report, we demonstrate that *C. neoformans* hyaluronic acid interacts with CD44 on HBMEC. Our results suggest that HBMEC CD44 is a primary receptor during *C. neoformans* infection, based on the following observations. First, anti-CD44 neutralizing antibody treatment was able to significantly reduce *C. neoformans* association with HBMEC. Second, *C. neoformans* association was considerably impaired using either CD44-knock-down HBMEC or *C. neoformans* hyaluronic acid-deficient strains. Third, overexpression of CD44 in HBMEC increased their association activity towards *C. neoformans*. Fourth, confocal microscopic images showed that CD44 was enriched at and around the *C. neoformans* association sites. Fifth, upon *C. neoformans* and HBMEC engagement, a subpopulation of CD44 and actin

translocated to the host membrane rafts. Our results highlight the interactions between *C. neoformans* hyaluronic acid and host CD44 and the dynamic results of these interactions, which may represent events during the adhesion and entry of *C. neoformans* at HBMEC membrane rafts.

3.1091 Evidence for a Superoxide Permeability Pathway in Endosomal Membranes
Mumbengegwi, D.R., Li, Q., Li, C., Bear, C.E. and Engelhardt, J.F.
Mol. Cell. Biol., ***28(11)***, *3700-3712 (2008)*

The compartmentalized production of superoxide ($\cdot O_2^-$) by endosomal NADPH oxidase is important in the redox-dependent activation of NF-κB following interleukin 1β (IL-1β) stimulation. It remains unclear how $\cdot O_2^-$ produced within endosomes facilitates redox-dependent signaling events in the cytoplasm. We evaluated $\cdot O_2^-$ movement out of IL-1β-stimulated endosomes and whether SOD1 at the endosomal surface mediates redox-signaling events required for NF-κB activation. The relative outward permeability of NADPH-dependent $\cdot O_2^-$ from fractionated endosomes was assessed using membrane-permeable (luminol and lucigenin) and -impermeable (isoluminol) luminescent probes for $\cdot O_2^-$. In these studies, ~60% of $\cdot O_2^-$ efflux out of endosomes was inhibited by treatment with either of two anion channel blockers, 4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) or niflumic acid (NFA). Furthermore, radioisotopic electrodiffusion flux assays on endomembrane proteoliposomes suggested that $\cdot O_2^-$ and Cl^- are transported through the same DIDS-sensitive channel(s). Rab5-based immunoaffinity isolation of IL-1β-stimulated early endosomes demonstrated SOD1 recruitment to endosomes harboring the IL-1 receptor. Finally, SOD1-deficient cells were found to be defective in their ability to activate NF-κB following IL-1β stimulation. Together, these results suggest that $\cdot O_2^-$ exits endosomes through a DIDS-sensitive chloride channel(s) and that SOD1-mediated dismutation of $\cdot O_2^-$ at the endosomal surface may produce the localized H_2O_2 required for redox-activation of NF-κB.

3.1092 Targeting of Pseudorabies Virus Structural Proteins to Axons Requires Association of the Viral Us9 Protein with Lipid Rafts
Lyman, M.G., Curanovic, D. And Enquist, L.W.
PlosPathogens, ***4(5)***, *e1000065*

The pseudorabies virus (PRV) Us9 protein plays a central role in targeting viral capsids and glycoproteins to axons of dissociated sympathetic neurons. As a result, Us9 null mutants are defective in anterograde transmission of infection *in vivo*. However, it is unclear how Us9 promotes axonal sorting of so many viral proteins. It is known that the glycoproteins gB, gC, gD and gE are associated with lipid raft microdomains on the surface of infected swine kidney cells and monocytes, and are directed into the axon in a Us9-dependent manner. In this report, we determined that Us9 is associated with lipid rafts, and that this association is critical to Us9-mediated sorting of viral structural proteins. We used infected non-polarized and polarized PC12 cells, a rat pheochromocytoma cell line that acquires many of the characteristics of sympathetic neurons in the presence of nerve growth factor (NGF). In these cells, Us9 is highly enriched in detergent-resistant membranes (DRMs). Moreover, reducing the affinity of Us9 for lipid rafts inhibited anterograde transmission of infection from sympathetic neurons to epithelial cells *in vitro*. We conclude that association of Us9 with lipid rafts is key for efficient targeting of structural proteins to axons and, as a consequence, for directional spread of PRV from pre-synaptic to post-synaptic neurons and cells of the mammalian nervous system.

3.1093 Cholesterol Substitution Increases the Structural Heterogeneity of Caveolae
Jansen, M. et al
J. Biol. Chem., ***283(21)***, *14610-14618 (2008)*

Caveolin-1 binds cholesterol and caveola formation involves caveolin-1 oligomerization and cholesterol association. The role of cholesterol in caveolae has so far been addressed by methods that compromise membrane integrity and abolish caveolar invaginations. To study the importance of sterol specificity for the structure and function of caveolae, we replaced cholesterol in mammalian cells with its immediate precursor desmosterol by inhibiting 24-dehydrocholesterol reductase. Desmosterol could substitute for cholesterol in maintaining cell growth, membrane integrity, and preserving caveolar invaginations. However, in desmosterol cells the affinity of caveolin-1 for sterol and the stability of caveolin oligomers were decreased. Moreover, caveolar invaginations became more heterogeneous in dimensions and in the number of caveolin-1 molecules per caveola. Despite the altered caveolar structure, caveolar ligand uptake was only moderately inhibited. We found that in desmosterol cells, Src kinase phosphorylated Cav1 at Tyr^{14} more avidly than in cholesterol cells. Taken the role of Cav1 Tyr^{14} phosphorylation in caveolar

endocytosis, this may help to preserve caveolar uptake in desmosterol cells. We conclude that a sterol C24 double bond interferes with caveolin-sterol interaction and perturbs caveolar morphology but facilitates Cav1 Src phosphorylation and allows caveolar endocytosis. More generally, substitution of cholesterol by a structurally closely related sterol provides a method to selectively modify membrane protein-sterol affinity, structure and function of cholesterol-dependent domains without compromising membrane integrity.

3.1094 Synaptic Vesicles Are Constitutively Active Fusion Machines that Function Independently of Ca^{2+}
Holt, M., Riedel, D., Stein, A., Schuette, C. and Jahn, R.
*Current Biology, **18**, 715-722 (2008)*

Background
In neurons, release of neurotransmitter occurs through the fusion of synaptic vesicles with the plasma membrane. Many proteins required for this process have been identified, with the SNAREs syntaxin 1, SNAP-25, and synaptobrevin thought to constitute the core fusion machinery. However, there is still a large gap between our understanding of individual protein-protein interactions and the functions of these proteins revealed by perturbations in intact synaptic preparations. To bridge this gap, we have used purified synaptic vesicles, together with artificial membranes containing coreconstituted SNAREs as reaction partners, in fusion assays.
Results
By using complementary experimental approaches, we show that synaptic vesicles fuse constitutively, and with high efficiency, with proteoliposomes containing the plasma membrane proteins syntaxin 1 and SNAP-25. Fusion is inhibited by clostridial neurotoxins and involves the formation of SNARE complexes. Despite the presence of endogenous synaptotagmin, Ca^{2+} does not enhance fusion, even if phosphatidylinositol 4,5-bisphosphate is present in the liposome membrane. Rather, fusion kinetics are dominated by the availability of free syntaxin 1/SNAP-25 acceptor sites for synaptobrevin.
Conclusions
Synaptic vesicles are constitutively active fusion machines, needing only synaptobrevin for activity. Apparently, the final step in fusion does not involve the regulatory activities of other vesicle constituents, although these may be involved in regulating earlier processes. This is particularly relevant for the calcium-dependent regulation of exocytosis, which, in addition to synaptotagmin, requires other factors not present in the vesicle membrane. The in vitro system described here provides an ideal starting point for unraveling of the molecular details of such regulatory events.

3.1095 Caveolae structure and function
Thomas, C.M. and Smart, E.J.
*J. Cell. Mol. Med., **12(3)**, 796-809 (2008)*

Studies on the structure and function of caveolae have revealed how this versatile subcellular organelle can influence numerous signalling pathways. This brief review will discuss a few of the key features of caveolae as it relates to signalling and disease processes.

3.1096 Segregation and rapid turnover of EDEM1 by an autophagy-like mechanism modulates standard ERAD and folding activities
Cali, T., Galli, C., Olivari, S. And Molinari, M.
*Biochem. Biophys. Res. Comm., **371**, 405-410 (2008)*

EDEM1 is a crucial regulator of endoplasmic reticulum (ER)-associated degradation (ERAD) that extracts non-native glycopolypeptides from the calnexin chaperone system. Under normal growth conditions, the intralumenal level of EDEM1 must be low to prevent premature interruption of ongoing folding programs. We report that in unstressed cells, EDEM1 is segregated from the bulk ER into LC3-I-coated vesicles and is rapidly degraded. The rapid turnover of EDEM1 is regulated by a novel mechanism that shows similarities but is clearly distinct from macroautophagy. Cells with defective EDEM1 turnover contain unphysiologically high levels of EDEM1, show enhanced ERAD activity and are characterized by impaired capacity to efficiently complete maturation of model glycopolypeptides. We define as *ERAD tuning* the mechanisms operating in the mammalian ER at steady state to offer kinetic advantage to folding over disposal of unstructured nascent chains by selective and rapid degradation of ERAD regulators.

3.1097 Caveolin-1 Expression in Trabecular Meshwork Cells
Nolan, M.J. et al
*Invest. Ophthalmol. Vis. Sci., **49**, E-abstract 1627 (2008)*

Purpose: Caveolin-1, a 22-kDa, intergral membrane protein forming oligomers in cells, is a principal component of caveolae in lipid rafts and is also important in regulating vesicle trafficking through cells. Caveolin-1 is involved, consequently, in signal transduction, tight junction formation, endocytosis, transcytosis, as well serving as a mechanosensor in endothelial cells. Lipid-modified proteins such as endothelial nitric oxide synthase and the Src family of kinases can target to caveolae and interact with caveolin-1 to regulate signal transduction. The purpose of this study was to determine whether trabecular meshwork (TM) cells express caveolin-1 and if caveolin-1 expression is influenced by dexamethasone (Dex) treatment.
Methods: Human TM cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) until confluent, washed twice with PBS, and incubated in DMEM containing 0.1% FCS and 100 µM Dex for 1, 24 and 96 hours. The media was aspirated; the cells were washed with cold PBS, subjected to lysis buffer (Sigma CS0750) containing 1% Triton X-100, and separated by **Optiprep** density gradient (Sigma D1556). The preparation was centrifuged at 200,000 x g for 18 hrs; nine 0.5 ml fractions were pipetted from the top (lightest) to bottom (heaviest). Each fraction was analyzed for protein content, resolved by SDS polyarylamide electrophoresis, and immunoblotted with mouse monoclonal anti-caveolin-1 antibody (Sigma).
Results: The distribution of 22-kDa caveolin-1 in TM cells was in fractions 4 through 7 and the strongest intensity was in fraction 5; a high molecular weight (>300-kDa) was observed in fractions 5 through 7. The distribution of 22-kDa caveolin-1 in Dex-treated TM cells was in fractions 5 through 7 and the strongest intensity was in fractions 5 and 6, with minor amounts in fraction 8 and 9; a high molecular weight (>300-kDa) was also observed in fractions 5 through 7. The amount of caveolin-1 in Dex-treated TM cells was considerably greater, approximately two-fold. Similarly, the amount of caveolin-1 in the media was also increased in the Dex-treated TM cells.
Conclusions: This is the first demonstration of caveolin-1 in TM cells. Dex treatment increased the cell caveolin-1 content and changed caveolin-1 density gradient distribution as well as increasing the secretion of media caveolin-1. These results indicate that caveolin-1 may play an important role in TM barrier function, cell signaling, and endocytosis.

3.1098 Overexpression of a host factor TOM1 inhibits tomato mosaic virus propagation and suppression of RNA silencing
Hagiwara-Komoda, Y. et al
*Virology, **376**, 132-139 (2008)*

A plant integral membrane protein TOM1 is involved in the multiplication of *Tomato mosaic virus* (ToMV). TOM1 interacts with ToMV replication proteins and has been suggested to tether the replication proteins to the membranes where the viral RNA synthesis takes place. We have previously demonstrated that inactivation of *TOM1* results in reduced ToMV multiplication. In the present study, we show that overexpression of TOM1 in tobacco also inhibits ToMV propagation. TOM1 overexpression led to a decreased accumulation of the soluble form of the replication proteins and interfered with the ability of the replication protein to suppress RNA silencing. The reduced accumulation of the soluble replication proteins was also observed in a silencing suppressor-defective ToMV mutant. Based on these results, we propose that RNA silencing suppression is executed by the soluble form of the replication proteins and that efficient ToMV multiplication requires balanced accumulation of the soluble and membrane-bound replication proteins.

3.1099 The human SIRT3 protein deacetylase is exclusively mitochondrial
Cooper, H.M: and Spelbrink, J.N.
*Biochem. J., **411**, 279-285 (2008)*

It has recently been suggested that perhaps as many as 20% of all mitochondrial proteins are regulated through lysine acetylation while SIRT3 has been implicated as an important mitochondrial protein deacetylase. It is therefore of crucial importance that the mitochondrial localization of potential protein deacetylases is unambiguously established. Although mouse SIRT3 was recently shown to be mitochondrial, HsSIRT3 (human SIRT3) was reported to be both nuclear and mitochondrial and to relocate from the nucleus to the mitochondrion upon cellular stress. In the present study we show, using various HsSIRT3 expression constructs and a combination of immunofluorescence and careful subcellular

fractionation, that in contrast with earlier reports HsSIRT3 is exclusively mitochondrial. We discuss possible experimental explanations for these discrepancies. In addition we suggest, on the basis of the analysis of public genome databases, that the full-length mouse SIRT3 protein is a 37 kDa mitochondrial precursor protein contrary to the previously suggested 29 kDa protein.

3.1100 CLN3p Impacts Galactosylceramide Transport, Raft Morphology, and Lipid Content
Rusyn, E., Mousallem, T., Persaud-Sawin, D-A., Miller, S. and Boustany, R-M.N.
*Pediatr. Res., **63(6)**, 625-631 (2008)*

Juvenile neuronal ceroid lipofuscinosis (JNCL) belongs to the neuronal ceroid lipofuscinoses characterized by blindness/seizures/motor/cognitive decline and early death. JNCL is caused by CLN3 gene mutations that negatively modulate cell growth/apoptosis. CLN3 protein (CLN3p) localizes to Golgi/Rab4-/Rab11-positive endosomes and lipid rafts, and harbors a galactosylceramide (GalCer) lipid raft-binding domain. Goals are proving CLN3p participates in GalCer transport from Golgi to rafts, and GalCer deficits negatively affect cell growth/apoptosis. GalCer/mutant CLN3p are retained in Golgi, with CLN3p rescuing GalCer deficits in rafts. Diminishing GalCer in normal cells by GalCer synthase siRNA negatively affects cell growth/apoptosis. GalCer restores JNCL cell growth. WT CLN3p binds GalCer, but not mutant CLN3p. Sphingolipid content of rafts/Golgi is perturbed with diminished GalCer in rafts and accumulation in Golgi. CLN3-deficient raft vesicular structures are small by transmission electron microscopy, reflecting altered sphingolipid composition of rafts. CLN1/CLN2/CLN6 proteins bind to lysophosphatidic acid/sulfatide, CLN6/CLN8 proteins to GalCer, and CLN8 protein to ceramide. Sphingolipid composition/morphology of CLN1-/CLN2-/CLN6-/CLN8- and CLN9-deficient rafts are altered suggesting changes in raft structure/lipid stoichiometry could be common themes underlying these diseases.

3.1101 Lovastatin inhibits amyloid precursor protein (APP) [beta]-cleavage through reduction of APP distribution in Lubrol WX extractable low density lipid rafts
Won, J-S. et al
*J. Neurochem., **105**, 1536-1549 (2008)*

Previous studies have described that statins (inhibitors of cholesterol and isoprenoid biosynthesis) inhibit the output of amyloid-[beta] (A[beta]) in the animal model and thus decrease risk of Alzheimer's disease. However, their action mechanism(s) in A[beta] precursor protein (APP) processing and A[beta] generation is not fully understood. In this study, we report that lovastatin treatment reduced A[beta] output in cultured hippocampal neurons as a result of reduced APP levels and [beta]-secretase activities in low density Lubrol WX (non-ionic detergent) extractable lipid rafts (LDLR). Rather than altering cholesterol levels in lipid raft fractions and thus disrupting lipid raft structure, lovastatin decreased A[beta] generation through down-regulating geranylgeranyl-pyrophosphate dependent endocytosis pathway. The inhibition of APP endocytosis by treatment with lovastatin and reduction of APP levels in LDLR fractions by treatment with phenylarsine oxide (a general endocytosis inhibitor) support the involvement of APP endocytosis in APP distribution in LDLR fractions and subsequent APP [beta]-cleavage. Moreover, lovastatin-mediated down-regulation of endocytosis regulators, such as early endosomal antigen 1, dynamin-1, and phosphatidylinositol 3-kinase activity, indicates that lovastatin modulates APP endocytosis possibly through its pleiotropic effects on endocytic regulators. Collectively, these data report that lovastatin mediates inhibition of LDLR distribution and [beta]-cleavage of APP in a geranylgeranyl-pyrophosphate and endocytosis-dependent manner.

3.1102 Synaptotagmin VII Regulates Bone Remodeling by Modulating Osteoclast and Osteoblast Secretion
Zhao, H. et al
*Developmental Cell, **14**, 914-925 (2008)*

Maintenance of bone mass and integrity requires a tight balance between resorption by osteoclasts and formation by osteoblasts. Exocytosis of functional proteins is a prerequisite for the activity of both cells. In the present study, we show that synaptotagmin VII, a calcium sensor protein that regulates exocytosis, is associated with lysosomes in osteoclasts and bone matrix protein-containing vesicles in osteoblasts. Absence of synaptotagmin VII inhibits cathepsin K secretion and formation of the ruffled border in osteoclasts and bone matrix protein deposition in osteoblasts, without affecting the differentiation of either cell. Reflecting these in vitro findings, synaptotagmin VII-deficient mice are osteopenic due to impaired bone resorption and formation. Therefore, synaptotagmin VII plays an important role in bone remodeling and homeostasis by modulating secretory pathways functionally important in osteoclasts and osteoblasts.

3.1103 Multimerization of Tegument Protein pp28 within the Assembly Compartment Is Required for Cytoplasmic Envelopment of Human Cytomegalovirus
Seo, J-Y. and Britt, W.J.
*J. Virol., **82(13)**, 6272-7287 (2008)*

Human cytomegalovirus (HCMV) UL99-encoded pp28 is an essential tegument protein required for envelopment and production of infectious virus. Nonenveloped virions accumulate in the cytoplasm of cells infected with recombinant viruses with the UL99 gene deleted. Previous results have suggested that a key function of pp28 in the envelopment of infectious HCMV is expressed after the protein localizes in the assembly compartment (AC). In this study, we investigated the potential role of pp28 multimerization in the envelopment of the infectious virion. Our results indicated that pp28 multimerized during viral infection and that interacting domains responsible for self-interaction were localized in the amino terminus of the protein (amino acids [aa] 1 to 43). The results from transient-expression and/or infection assays indicated that the self-interaction took place in the AC. A mutant pp28 molecule containing only the first 35 aa failed to accumulate in the AC, did not interact with pp28 in the AC, and could not support virus replication. In contrast, the first 50 aa of pp28 was sufficient for the self-interaction within the AC and the assembly of infectious virus. Recombinant viruses encoding an in-frame deletion of aa 26 to 33 of pp28 were replication competent, whereas infectious virus was not recovered from HCMV BACs lacking aa 26 to 43. These findings suggested that the accumulation of pp28 was a prerequisite for multimerization of pp28 within the AC and that pp28 multimerization in the AC represented an essential step in the envelopment and production of infectious virions.

3.1104 Functional stabilization of Kv1.5 protein by Hsp70 in mammalian cell lines
Hirota, Y. et al
*Biochem. Biophys. Acta., **372**, 469-474 (2008)*

The aim of this study was to elucidate the mechanisms for regulations of cardiac Kv1.5 channel expression. We particularly focused on the role of heat shock proteins (Hsps). We tested the effects of Hsps on the stability of Kv1.5 channels using biochemical and electrophysiological techniques: co-expression of Kv1.5 and Hsp family proteins in mammalian cell lines, followed by Western blotting, immunoprecipitation, pulse-chase analysis, immunofluorescence and whole-cell patch clamp. Hsp70 and heat shock factor 1 increased the expression of Kv1.5 protein in HeLa and COS7 cells, whereas either Hsp40, 27 or 90 did not. Hsp70 prolonged the half-life of Kv1.5 protein. Hsp70 was co-immunoprecipitated and co-localized with Kv1.5-FLAG. Hsp70 significantly increased the immunoreactivity of Kv1.5 in the endoplasmic reticulum, Golgi apparatus and on the cell membrane. Hsp70 enhanced Kv1.5 current of transfected cells, which was abolished by pretreatment with brefeldin A or colchicine. Thus, Hsp70, but not other Hsps, stabilizes functional Kv1.5 protein.

3.1105 Sphingomyelin Functions as a Novel Receptor for Helicobacter pylori VacA
Gupta, V.R. et al
*PLOSpathogens, **4(5)**, e1000073 (2008)*

The vacuolating cytotoxin (VacA) of the gastric pathogen *Helicobacter pylori* binds and enters epithelial cells, ultimately resulting in cellular vacuolation. Several host factors have been reported to be important for VacA function, but none of these have been demonstrated to be essential for toxin binding to the plasma membrane. Thus, the identity of cell surface receptors critical for both toxin binding and function has remained elusive. Here, we identify VacA as the first bacterial virulence factor that exploits the important plasma membrane sphingolipid, sphingomyelin (SM), as a cellular receptor. Depletion of plasma membrane SM with sphingomyelinase inhibited VacA-mediated vacuolation and significantly reduced the sensitivity of HeLa cells, as well as several other cell lines, to VacA. Further analysis revealed that SM is critical for VacA interactions with the plasma membrane. Restoring plasma membrane SM in cells previously depleted of SM was sufficient to rescue both toxin vacuolation activity and plasma membrane binding. VacA association with detergent-resistant membranes was inhibited in cells pretreated with SMase C, indicating the importance of SM for VacA association with lipid raft microdomains. Finally, VacA bound to SM in an *in vitro* ELISA assay in a manner competitively inhibited by lysenin, a known SM-binding protein. Our results suggest a model where VacA may exploit the capacity of SM to preferentially partition into lipid rafts in order to access the raft-associated cellular machinery previously shown to be required for toxin entry into host cells.

3.1106 Mammalian cell expression of an active site mutant of Pseudomonas exotoxin disrupts LRP1 maturation

Pastrana, D.V., Yun, C.H., McKee, M.L. and FitzGerald, D.J.
J. Biomed. Sci., ***15***, *427-439 (2008)*

Low density lipoprotein receptor-related protein 1, (LRP1) is a large multifunctional receptor that binds more than 25 physiologic ligands. In addition, it functions as the surface receptor for several Rhinoviruses, HIV-tat and Pseudomonas exotoxin (PE). We report that the expression of PE within mammalian cells can serve as a probe of LRP1 maturation and functionality. To avoid cell killing, an enzymatically inactive form of the toxin (PEΔ553) was expressed. A permanent cell line (termed CY301) was established whereby PEΔ553 was expressed continually into the ER of CHO cells. CY301 cells were 100-fold resistant to exogenously added active PE but exhibited no cross-resistance to other toxins. Our studies indicate that PEΔ553 bound to immature LRP1 in the ER, prevented its maturation to the cell surface and thereby produced a toxin resistant phenotype. By confocal microscopy, cell-associated PEΔ553 was localized to the ER and co-localized with LRP1. Further characterization of CY301 cells indicated that RAP, the chaperone that aids in LRP1 folding, was released to the growth media. Thus the intracellular expression of PEΔ553 appears to be a valuable probe of LRP1 maturation and trafficking.

3.1107 Engineered Bacterial Outer Membrane Vesicles with Enhanced Functionality

Kim, J-Y. et al
J. Mol. Biol., ***380***, *51-66 (2008)*

We have engineered bacterial outer membrane vesicles (OMVs) with dramatically enhanced functionality by fusing several heterologous proteins to the vesicle-associated toxin ClyA of *Escherichia coli*. Similar to native unfused ClyA, chimeric ClyA fusion proteins were found localized in bacterial OMVs and retained activity of the fusion partners, demonstrating for the first time that ClyA can be used to co-localize fully functional heterologous proteins directly in bacterial OMVs. For instance, fusions of ClyA to the enzymes β-lactamase and organophosphorus hydrolase resulted in synthetic OMVs that were capable of hydrolyzing β-lactam antibiotics and paraoxon, respectively. Similarly, expression of an anti-digoxin single-chain Fv antibody fragment fused to the C terminus of ClyA resulted in designer “immuno-MVs” that could bind tightly and specifically to the antibody's cognate antigen. Finally, OMVs displaying green fluorescent protein fused to the C terminus of ClyA were highly fluorescent and, as a result of this new functionality, could be easily tracked during vesicle interaction with human epithelial cells. We expect that the relative plasticity exhibited by ClyA as a fusion partner should prove useful for: (i) further mechanistic studies to identify the vesiculation machinery that regulates OMV secretion and to map the intracellular routing of ClyA-containing OMVs during invasion of host cells; and (ii) biotechnology applications such as surface display of proteins and delivery of biologics.

3.1108 Cholesterol Depletion Reduces Helicobacter pylori CagA Translocation and CagA-Induced Responses in AGS Cells

Lai, C-H. et al
Infect. Immun., ***76(7)***, *3293-3303 (2008)*

Infection with *Helicobacter pylori cagA*-positive strains is associated with gastritis, ulcerations, and gastric cancer. CagA is translocated into infected epithelial cells by a type IV secretion system and can be tyrosine phosphorylated, inducing signal transduction and motogenic responses in epithelial cells. Cellular cholesterol, a vital component of the membrane, contributes to membrane dynamics and functions and is important in VacA intoxication and phagocyte evasion during *H. pylori* infection. In this investigation, we showed that cholesterol extraction by methyl-β-cyclodextrin reduced the level of CagA translocation and phosphorylation. Confocal microscope visualization revealed that a significant portion of translocated CagA was colocalized with the raft marker GM1 and c-Src during infection. Moreover, GM1 was rapidly recruited into sites of bacterial attachment by live-cell imaging analysis. CagA and VacA were cofractionated with detergent-resistant membranes (DRMs), suggesting that the distribution of CagA and VacA is associated with rafts in infected cells. Upon cholesterol depletion, the distribution shifted to non-DRMs. Accordingly, the CagA-induced hummingbird phenotype and interleukin-8 induction were blocked by cholesterol depletion. Raft-disrupting agents did not influence bacterial adherence but did significantly reduce internalization activity in AGS cells. Together, these results suggest that delivery of CagA into epithelial cells by the bacterial type IV secretion system is mediated in a cholesterol-dependent manner.

3.1109 Rhesus lymphocryptovirus latent membrane protein 2A activates β-catenin signaling and inhibits differentiation in epithelial cells
Siler, C.A. and Raab-Traub, N.
*Virology, **377**, 273-279 (2008)*

Rhesus lymphocryptovirus (LCV) is a γ-herpesvirus closely related to Epstein–Barr virus (EBV). The rhesus latent membrane protein 2A (LMP2A) is highly homologous to EBV LMP2A. EBV LMP2A activates the phosphatidylinositol 3-kinase (PI3K) and β-catenin signaling pathways in epithelial cells and affects differentiation. In the present study, the biochemical and biological properties of rhesus LMP2A in epithelial cells were investigated. The expression of rhesus LMP2A in epithelial cells induced Akt activation, GSK3β inactivation and accumulation of β-catenin in the cytoplasm and nucleus. The nuclear translocation, but not accumulation of β-catenin was dependent on Akt activation. Rhesus LMP2A also impaired epithelial cell differentiation; however, this process was not dependent upon Akt activation. A mutant rhesus LMP2A lacking six transmembrane domains functioned similarly to wild-type rhesus LMP2A indicating that the full number of transmembrane domains is not required for effects on β-catenin or cell differentiation. These results underscore the similarity of LCV to EBV and the suitability of the macaque as an animal model for studying EBV pathogenesis.

3.1110 Canine MDCK cell lines are refractory to infection with human and mouse prions
Polymenidou, M. et al
*Vaccine, **26**, 2601-2614 (2008)*

Influenza vaccine production in embryonated eggs is associated with many disadvantages, and production in cell culture systems is a viable alternative. Madin Darby canine kidney (MDCK) cells are permissive for a variety of orthomyxoviruses and have proven particularly suitable for vaccine mass production. However, mammalian cells harboring the *Prnp* gene can theoretically acquire prion infections. Here, we have attempted to infect MDCK cells and substrains thereof with prions. We found that MDCK cells did not produce any protease-resistant PrP^{Sc} upon exposure to brain homogenates derived from humans suffering from Creutzfeldt–Jakob disease (CJD) or from mice infected with Rocky Mountain Laboratory (RML) scrapie prions. Further, transmission of MDCK lysates to N2aPK1 cells did not induce formation of PrP^{Sc} in the latter. PrP^{C} biogenesis and processing in MDCK cells were similar to those of prion-sensitive N2aPK1 cells. However, steady-state levels of PrP^{C} were very low, and PrP^{C} did not partition with detergent-resistant membranes upon density gradient analysis. These factors may account for their resistance to infection. Alternatively, prion resistance may be related to the specific sequence of canine *Prnp*, as suggested by the lack of documented prion diseases in dogs.

3.1111 Differential loss of cytochrome-c oxidase subunits in ischemia-reperfusion injury: exacerbation of COI subunit loss by PKC-ε inhibition
Yu, Q., Nguyen, T., Ogbi, M., Caldqwell, R.W. and Johnson, J.A.
*Am. J. Physiol. Heart Circ. Physiol., **294**, H2637-H2645 (2008)*

We have previously described a PKC-ε interaction with cytochrome oxidase subunit IV (COIV) that correlates with enhanced CO activity and cardiac ischemic preconditioning (PC). We therefore investigated the effects of PC and ischemia-reperfusion (I/R) injury on CO subunit levels in an anesthetized rat coronary ligation model. Homogenates prepared from the left ventricular regions at risk (RAR) and not at risk (RNAR) for I/R injury were fractionated into cell-soluble (S), 600 *g* low-speed centrifugation (L), gradient-purified mitochondrial (M), and 100,000 *g* particulate (P) fractions. In RAR tissue, PC (2 cycles of 5-min ischemia and 5-min reperfusion) decreased the COI in the P fraction (~29% of total cellular COI), suggesting changes in interfibrillar mitochondria. After 30 min of ischemia and 120 min of reperfusion, total COI levels decreased in the RAR by 72%. Subunit Va was also downregulated by 42% following prolonged I/R in the RAR. PC administered before I/R reduced the loss of COI in the M and P fractions ~30% and prevented COVa losses completely. We observed no losses in subunits Vb and VIIa following I/R alone; however, significant losses occurred when PC was administered before prolonged I/R. Delivery of a cell-permeable PKC-ε translocation inhibitor (εV1-2) to isolated rat hearts before prolonged I/R dramatically increased COI loss, suggesting that PKC-ε protects COI levels. We propose that additional measures to protect CO subunits when coadministered with PC may improve its cardioprotection against I/R injury.

3.1112 CD20 Homo-oligomers Physically Associate with the B Cell Antigen Receptor: DISSOCIATION UPON RECEPTOR ENGAGEMENT AND RECRUITMENT OF PHOSPHOPROTEINS AND CALMODULIN-BINDING PROTEINS

Polyak, M.J., Li, H., Shariat, N. And Deans, J.P.
J. Biol. Chem., ***283(27)***, *18545-18552 (2008)*

B cell antigen receptor (BCR) signaling initiates sustained cellular calcium influx necessary for the development, differentiation, and activation of B lymphocytes. CD20 is a B cell-restricted tetraspanning protein organized in the plasma membrane as multimeric molecular complexes involved in BCR-activated calcium entry. Using coprecipitation of native CD20 with tagged or truncated forms of the molecule, we provide here direct evidence of CD20 homo-oligomerization into tetramers. Additionally, the function of CD20 was explored by examining its association with surface-labeled and intracellular proteins before and after BCR signaling. Two major surface-labeled proteins that coprecipitated with CD20 were identified as the heavy and light chains of cell surface IgM, the antigen-binding components of the BCR. After activation, BCR-CD20 complexes dissociated, and phosphoproteins and calmodulin-binding proteins were transiently recruited to CD20. These data provide new evidence of the involvement of CD20 in signaling downstream of the BCR and, together with the previously described involvement of CD20 in calcium influx, the first evidence of physical coupling of the BCR to a calcium entry pathway.

3.1113 The Subcellular Distribution of Calnexin Is Mediated by PACS-2

Myhill, N. et aql
Mol. Cell Biol., ***19***, *2777-2788 (2008)*

Calnexin is an endoplasmic reticulum (ER) lectin that mediates protein folding on the rough ER. Calnexin also interacts with ER calcium pumps that localize to the mitochondria-associated membrane (MAM). Depending on ER homeostasis, varying amounts of calnexin target to the plasma membrane. However, no regulated sorting mechanism is so far known for calnexin. Our results now describe how the interaction of calnexin with the cytosolic sorting protein PACS-2 distributes calnexin between the rough ER, the MAM, and the plasma membrane. Under control conditions, more than 80% of calnexin localizes to the ER, with the majority on the MAM. PACS-2 knockdown disrupts the calnexin distribution within the ER and increases its levels on the cell surface. Phosphorylation by protein kinase CK2 of two calnexin cytosolic serines (Ser554/564) reduces calnexin binding to PACS-2. Consistent with this, a Ser554/564 ⟶ Asp phosphomimic mutation partially reproduces PACS-2 knockdown by increasing the calnexin signal on the cell surface and reducing it on the MAM. PACS-2 knockdown does not reduce retention of other ER markers. Therefore, our results suggest that the phosphorylation state of the calnexin cytosolic domain and its interaction with PACS-2 sort this chaperone between domains of the ER and the plasma membrane.

3.1114 Triglyceride-rich lipoprotein lipolysis increases aggregation of endothelial cell membrane microdomains and produces reactive oxygen species

Wang, L., Sapuri-Butti, A.R., Aung, H.H., Parikh, A.N. and Rutlegde, J.C.
Am. J. Physiol. Heart Circ. Physiol., ***295***, *H237-H244 (2008)*

Triglyceride-rich lipoprotein (TGRL) lipolysis may provide a proinflammatory stimulus to endothelium. Detergent-resistant plasma membrane microdomains (lipid rafts) have a number of functions in endothelial cell inflammation. The mechanisms of TGRL lipolysis-induced endothelial cell injury were investigated by examining endothelial cell lipid rafts and production of reactive oxygen species (ROS). Lipid raft microdomains in human aortic endothelial cells were visualized by confocal microscopy with fluorescein isothiocyanate-labeled cholera toxin B as a lipid raft marker. Incubation of Atto565-labeled TGRL with lipid raft-labeled endothelial cells showed that TGRL colocalized with the lipid rafts, TGRL lipolysis caused clustering and aggregation of lipid rafts, and colocalization of TGRL remnant particles on the endothelial cells aggregated lipid rafts. Furthermore, TGRL lipolysis caused translocation of low-density lipoprotein receptor-related protein, endothelial nitric oxide synthase, and caveolin-1 from raft regions to nonraft regions of the membrane 3 h after treatment with TGRL lipolysis. TGRL lipolysis significantly increased the production of ROS in endothelial cells, and both NADPH oxidase and cytochrome *P*-450 inhibitors reduced production of ROS. Our studies suggest that alteration of lipid raft morphology and composition and ROS production could contribute to TGRL lipolysis-mediated endothelial cell injury.

3.1115 Characterization of seipin/BSCL2, a protein associated with spastic paraplegia 17

Ito, D., Fujisawa, T., Iida, H and Suzuki, N.
Neurobiol. Disease, ***31(2)***, *266-277 (2008)*

Seipin, which is encoded by the *BSCL2* gene, is a glycoprotein of unknown biochemical function that is associated with dominant hereditary motor neuron diseases. Mutations in the *N*-glycosylation site of seipin are associated with the disease states and result in accumulation of unfolded protein in the endoplasmic reticulum (ER), leading to the unfolded protein response (UPR) and cell death, suggesting that these diseases are tightly associated with ER stress. Here, we determined the subcellular localization, functional domains, and distribution of seipin in tissues. Our studies show that the transmembrane domains in seipin are critical for ER retention, ubiquitination, formation of inclusions, and activation of UPR. Using immunohistochemistry, seipin expression is detected in neurons in the spinal cord and in the frontal lobe cortex of the brain. The present study provides new insights into the biology of seipin protein that should help our understanding of the pathogenesis of seipin-related diseases.

3.1116 EGF induces coalescence of different lipid rafts
Hofman, E.G. et al
*J. Cell Sci., **121**, 2519-2528 (2008)*

The suggestion that microdomains may function as signaling platforms arose from the presence of growth factor receptors, such as the EGFR, in biochemically isolated lipid raft fractions. To investigate the role of EGFR activation in the organization of lipid rafts we have performed FLIM analyses using putative lipid raft markers such as ganglioside GM1 and glycosylphosphatidylinositol (GPI)-anchored GFP (GPI-GFP). The EGFR was labeled using single domain antibodies from *Llama glama* that specifically bind the EGFR without stimulating its kinase activity. Our FLIM analyses demonstrate a cholesterol-independent colocalization of GM1 with EGFR, which was not observed for the transferrin receptor. By contrast, a cholesterol-dependent colocalization was observed for GM1 with GPI-GFP. In the resting state no colocalization was observed between EGFR and GPI-GFP, but stimulation of the cell with EGF resulted in the colocalization at the nanoscale level of EGFR and GPI-GFP. Moreover, EGF induced the enrichment of GPI-GFP in a detergent-free lipid raft fraction. Our results suggest that EGF induces the coalescence of the two types of GM1-containing microdomains that might lead to the formation of signaling platforms.

3.1117 Regulation of the V-ATPase along the Endocytic Pathway Occurs through Reversible Subunit Association and Membrane Localization
Lafourcade, C., Sobo, K., Kieffer-Jaquinod, S., Garin, J. and van der Goot, F.G.
*PloS One, **3(7)**, e2758 (2008)*

The lumen of endosomal organelles becomes increasingly acidic when going from the cell surface to lysosomes. Luminal pH thereby regulates important processes such as the release of internalized ligands from their receptor or the activation of lysosomal enzymes. The main player in endosomal acidification is the vacuolar ATPase (V-ATPase), a multi-subunit transmembrane complex that pumps protons from the cytoplasm to the lumen of organelles, or to the outside of the cell. The active V-ATPase is composed of two multi-subunit domains, the transmembrane V_0 and the cytoplasmic V_1. Here we found that the ratio of membrane associated V_1/Vo varies along the endocytic pathway, the relative abundance of V_1 being higher on late endosomes than on early endosomes, providing an explanation for the higher acidity of late endosomes. We also found that all membrane-bound V-ATPase subunits were associated with detergent resistant membranes (DRM) isolated from late endosomes, raising the possibility that association with lipid-raft like domains also plays a role in regulating the activity of the proton pump. In support of this, we found that treatment of cells with U18666A, a drug that leads to the accumulation of cholesterol in late endosomes, affected acidification of late endosome. Altogether our findings indicate that the activity of the vATPase in the endocytic pathway is regulated both by reversible association/dissociation and the interaction with specific lipid environments.

3.1118 Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death
Apostolou, A., Shen, Y., Liang, Y., Luo, J. and Fang, S.
*Exp. Cell Res., **314**, 2454-2467 (2008)*

The accumulation of misfolded proteins in the endoplasmic reticulum (ER) causes ER stress that initiates the unfolded protein response (UPR). UPR activates both adaptive and apoptotic pathways, which contribute differently to disease pathogenesis. To further understand the functional mechanisms of UPR,

we identified 12 commonly UPR-upregulated genes by expression microarray analysis. Here, we describe characterization of Armet/MANF, one of the 12 genes whose function was not clear. We demonstrated that the Armet/MANF protein was upregulated by various forms of ER stress in several cell lines as well as by cerebral ischemia of rat. Armet/MANF was localized in the ER and Golgi and was also a secreted protein. Silencing Armet/MANF by siRNA oligos in HeLa cells rendered cells more susceptible to ER stress-induced death, but surprisingly increased cell proliferation and reduced cell size. Overexpression of Armet/MANF inhibited cell proliferation and improved cell viability under glucose-free conditions and tunicamycin treatment. Based on its inhibitory properties for both proliferation and cell death we have demonstrated, Armet is, thus, a novel secreted mediator of the adaptive pathway of UPR.

3.1119 Lipoprotein binding preference of CD36 is altered by filipin treatment
Zhang, J., Chu, W. and Crandall, I.
*Lipids in Health and Disease, **7**, 23-31 (2008)*

The class B scavenger receptor CD36 binds multiple ligands, including oxidized and native lipoprotein species. CD36 and the related receptor SR-B1 have been localized to caveolae, domains that participate in cell signaling, transcytosis, and regulation of cellular cholesterol homeostasis. Previous work has indicated that the ligand preference of CD36 may depend on the cell type in which it is expressed. To determine if the presence or absence of caveolae is the determining factor for lipoprotein preference, we treated CHO-CD36 and C32 cells with filipin. Filipin treatment rapidly increased the binding capacity of CD36 for the native lipoproteins HDL and LDL, but did not affect the binding capacity of CD36 for oxidized LDL. Filipin treatment affected the distribution of caveolin and CD36 suggesting that the presence caveolae may modulate the ligand preference of CD36. However, its molecular mechanism how CD36 and caveolin interaction in regulating lipoprotein transport remains to be further studied.

3.1120 Interaction of Hepatitis C Virus Nonstructural Protein 5A with Core Protein Is Critical for the Production of Infectious Virus Particles
Masaki, T. et al
*J. Virol., **82(16)**, 7964-7976 (2008)*

Nonstructural protein 5A (NS5A) of the hepatitis C virus (HCV) possesses multiple and diverse functions in RNA replication, interferon resistance, and viral pathogenesis. Recent studies suggest that NS5A is involved in the assembly and maturation of infectious viral particles; however, precisely how NS5A participates in virus production has not been fully elucidated. In the present study, we demonstrate that NS5A is a prerequisite for HCV particle production as a result of its interaction with the viral capsid protein (core protein). The efficiency of virus production correlated well with the levels of interaction between NS5A and the core protein. Alanine substitutions for the C-terminal serine cluster in domain III of NS5A (amino acids 2428, 2430, and 2433) impaired NS5A basal phosphorylation, leading to a marked decrease in NS5A-core interaction, disturbance of the subcellular localization of NS5A, and disruption of virion production. Replacing the same serine cluster with glutamic acid, which mimics the presence of phosphoserines, partially preserved the NS5A-core interaction and virion production, suggesting that phosphorylation of these serine residues is important for virion production. In addition, we found that the alanine substitutions in the serine cluster suppressed the association of the core protein with viral genome RNA, possibly resulting in the inhibition of nucleocapsid assembly. These results suggest that NS5A plays a key role in regulating the early phase of HCV particle formation by interacting with core protein and that its C-terminal serine cluster is a determinant of the NS5A-core interaction.

3.1121 Novel Role of Presenilins in Maturation and Transport of Integrin β1
Zou, K. et al
*Biochemistry, **47**, 3370-3378 (2008)*

Presenilins (PSs) play important roles in modulating the trafficking and maturation of several membrane proteins. However, the target membrane proteins whose trafficking and maturation are regulated by PS are largely unknown. By characterizing PS-deficient fibroblasts, we found that integrin β1 maturation is promoted markedly in PS1 and PS2 double-deficient fibroblasts and moderately in PS1- or PS2-deficient fibroblasts; in contrast, nicastrin maturation is completely inhibited in PS1 and PS2 double-deficient fibroblasts. Subcellular fractionation analysis demonstrated that integrin β1 maturation is promoted in the Golgi apparatus. The mature integrin β1 with an increased expression level was delivered to the cell surface, which resulted in an increased cell surface expression level of mature integrin β1 in PS1 and PS2 double-deficient fibroblasts. PS1 and PS2 double-deficient fibroblasts exhibited an enhanced ability to

adhere to culture dishes coated with integrin β1 ligands, namely, fibronectin and laminin. The inhibition of γ-secretase activity enhances neither integrin β1 maturation nor the adhesion of wild-type cells. Moreover, PS deficiency also promoted the maturation of integrins α3 and α5 and the cell surface expression of integrin α3. Integrins α3 and α5 were coimmunoprecipitated with integrin β1, suggesting the formation of the functional heterodimers integrins α3β1 and α5β1. Note that integrin β1 exhibited features opposite those of nicastrin in terms of maturation and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus in PS1 and PS2 double-deficient fibroblasts. Our results therefore suggest that PS regulates the maturation of membrane proteins in opposite directions and cell adhesion by modulating integrin maturation.

3.1122 Conventional Kinesin Holoenzymes Are Composed of Heavy and Light Chain Homodimers
DeBoer, S.R. et al
*Biochemistry, **47**, 4535-4543 (2008)*

Conventional kinesin is a major microtubule-based motor protein responsible for anterograde transport of various membrane-bounded organelles (MBO) along axons. Structurally, this molecular motor protein is a tetrameric complex composed of two heavy (kinesin-1) chains and two light chain (KLC) subunits. The products of three kinesin-1 (kinesin-1A, -1B, and -1C, formerly KIF5A, -B, and -C) and two KLC (KLC1, KLC2) genes are expressed in mammalian nervous tissue, but the functional significance of this subunit heterogeneity remains unknown. In this work, we examine all possible combinations among conventional kinesin subunits in brain tissue. In sharp contrast with previous reports, immunoprecipitation experiments here demonstrate that conventional kinesin holoenzymes are formed of kinesin-1 homodimers. Similar experiments confirmed previous findings of KLC homodimerization. Additionally, no specificity was found in the interaction between kinesin-1s and KLCs, suggesting the existence of six variant forms of conventional kinesin, as defined by their gene product composition. Subcellular fractionation studies indicate that such variants associate with biochemically different MBOs and further suggest a role of kinesin-1s in the targeting of conventional kinesin holoenzymes to specific MBO cargoes. Taken together, our data address the combination of subunits that characterize endogenous conventional kinesin. Findings on the composition and subunit organization of conventional kinesin as described here provide a molecular basis for the regulation of axonal transport and delivery of selected MBOs to discrete subcellular locations.

3.1123 A Proteomics Approach to Membrane Trafficking
Groen, A.J., de Vries, S.C. and Lilley, K.S.
*Plant Physiol., **147**, 1584-1589 (2008)*

Membrane trafficking, including that of integral membrane proteins as well as peripherally associated proteins, appears to be a vital process common to all eukaryotes. An important element of membrane trafficking is to determine the protein composition of the various endomembrane compartments. A major issue with such a compositional analysis is the difficulty of having to distinguish between resident components involved in specific tasks and the proteins that are in transit through the endomembrane system. Examples of resident proteins include components of the SNARE complex used to target membrane vesicles to different locations in the cell. In the case of functionally important residents, one would expect such proteins to have a fairly precise subcellular localization. In the case of proteins "passing through" an endosomal compartment en route to a final destination, one would expect to find the proteins colocalizing with many membrane compartments.
As is evident from several *Update* articles in this issue, ambiguity exists when employing cytological techniques to identify specific endomembrane compartments, while markers identified based on homology may behave differently in plant cells. Therefore, a proteomics approach based on proteins that would traffic through various parts of the endomembrane system, such as plasma membrane (PM) receptors, would be a welcome addition to membrane-trafficking studies. PM receptors are highly dependent on correct trafficking for their eventual localization, their biological function, and finally their degradation, while recent evidence suggests that endocytosis of PM receptors is an integral part of their biological function.
In this review, first, a short update on endocytosis and endosomal trafficking in Arabidopsis (*Arabidopsis thaliana*) is provided. In this section, we emphasize trafficking of PM receptors as a proteomics tool by looking at how the PM receptors traffic in a time-dependent fashion in order to determine the relationship between different endosomal compartments. Second, we describe the recent progress in advanced

proteomics techniques such as localization of organelle proteins by isotope tagging (LOPIT), by which proteins are assigned to different endosomal compartments.

3.1124 Molecular Identification of a SNAP-25-Like SNARE Protein in Paramecium
Schilde, C., Lutter, K., Kissmehl, R. And Plattner, H.
*Eukaryot. Cell, **7(8)**, 1387-1402 (2008)*

Using database searches of the completed *Paramecium tetraurelia* macronuclear genome with the metazoan SNAP-25 homologues, we identified a single 21-kDa Qb/c-SNARE in this ciliated protozoan, named *P. tetraurelia* SNAP (PtSNAP), containing the characteristic dual heptad repeat SNARE motifs of SNAP-25. The presence of only a single Qb/c class SNARE in *P. tetraurelia* is surprising in view of the multiple genome duplications and the high number of SNAREs found in other classes of this organism. As inferred from the subcellular localization of a green fluorescent protein (GFP) fusion construct, the protein is localized on a variety of intracellular membranes, and there is a large soluble pool of PtSNAP. Similarly, the PtSNAP that is detected with a specific antibody in fixed cells is associated with a number of intracellular membrane structures, including food vacuoles, the contractile vacuole system, and the sites of constitutive endo- and exocytosis. Surprisingly, using gene silencing, we could not assign a role to PtSNAP in the stimulated exocytosis of dense core vesicles (trichocysts), but we found an increased number of food vacuoles in PtSNAP-silenced cells. In conclusion, we identify PtSNAP as a *Paramecium* homologue of metazoan SNAP-25 that shows several divergent features, like resistance to cleavage by botulinum neurotoxins.

3.1125 Reciprocal interaction with G-actin and tropomyosin is essential for aquaporin-2 trafficking
Noda, Y. et al
*J. Cell Biol., **182(3)**, 587-601 (2008)*

Trafficking of water channel aquaporin-2 (AQP2) to the apical membrane and its vasopressin and protein kinase A (PKA)–dependent regulation in renal collecting ducts is critical for body water homeostasis. We previously identified an AQP2 binding protein complex including actin and tropomyosin-5b (TM5b). We show that dynamic interactions between AQP2 and the actin cytoskeleton are critical for initiating AQP2 apical targeting. Specific binding of AQP2 to G-actin in reconstituted liposomes is negatively regulated by PKA phosphorylation. Dual color fluorescence cross-correlation spectroscopy reveals local AQP2 interaction with G-actin in live epithelial cells at single-molecule resolution. Cyclic adenosine monophosphate signaling and AQP2 phosphorylation release AQP2 from G-actin. In turn, AQP2 phosphorylation increases its affinity to TM5b, resulting in reduction of TM5b bound to F-actin, subsequently inducing F-actin destabilization. RNA interference–mediated knockdown and overexpression of TM5b confirm its inhibitory role in apical trafficking of AQP2. These findings indicate a novel mechanism of channel protein trafficking, in which the channel protein itself critically regulates local actin reorganization to initiate its movement.

3.1126 Sensitization to the Lysosomal Cell Death Pathway by Oncogene-Induced Down-regulation of Lysosome-Associated Membrane Proteins 1 and 2
Fehrenbacher, N. et al
*Cancer Res., **68(16)**, 6623-6633 (2008)*

Expression and activity of lysosomal cysteine cathepsins correlate with the metastatic capacity and aggressiveness of tumors. Here, we show that transformation of murine embryonic fibroblasts with *v-H-ras* or *c-src*^{Y527F} changes the distribution, density, and ultrastructure of the lysosomes, decreases the levels of lysosome-associated membrane proteins (LAMP-1 and LAMP-2) in an extracellular signal-regulated kinase (ERK)- and cathepsin-dependent manner, and sensitizes the cells to lysosomal cell death pathways induced by various anticancer drugs (i.e., cisplatin, etoposide, doxorubicin, and siramesine). Importantly, *K-ras* and *erbb2* elicit a similar ERK-mediated activation of cysteine cathepsins, cathepsin-dependent down-regulation of LAMPs, and increased drug sensitivity in human colon and breast carcinoma cells, respectively. Notably, reconstitution of LAMP levels by ectopic expression or by cathepsin inhibitors protects transformed cells against the lysosomal cell death pathway. Furthermore, knockdown of either *lamp1* or *lamp2* is sufficient to sensitize the cells to siramesine-induced cell death and photo-oxidation–induced lysosomal destabilization. Thus, the transformation-associated ERK-mediated up-regulation of cysteine cathepsin expression and activity leads to a decrease in the levels of LAMPs, which in turn contributes to the enhanced sensitivity of transformed cells to drugs that trigger lysosomal membrane permeabilization. These data indicate that aggressive cancers with high cysteine cathepsin levels are

especially sensitive to lysosomal cell death pathways and encourage the further development of lysosome-targeting compounds for cancer therapy.

3.1127 Intravesicular Calcium Release Mediates the Motion and Exocytosis of Secretory Organelles: A STUDY WITH ADRENAL CHROMAFFIN CELLS
Camacho, M., Machado, J.D., Alvarez, J. and Borges, R.
J. Biol. Chem., ***283(33)***, *22383-22389 (2008)*

Nonstructural protein 5A (NS5A) of the hepatitis C virus (HCV) possesses multiple and diverse functions in RNA replication, interferon resistance, and viral pathogenesis. Recent studies suggest that NS5A is involved in the assembly and maturation of infectious viral particles; however, precisely how NS5A participates in virus production has not been fully elucidated. In the present study, we demonstrate that NS5A is a prerequisite for HCV particle production as a result of its interaction with the viral capsid protein (core protein). The efficiency of virus production correlated well with the levels of interaction between NS5A and the core protein. Alanine substitutions for the C-terminal serine cluster in domain III of NS5A (amino acids 2428, 2430, and 2433) impaired NS5A basal phosphorylation, leading to a marked decrease in NS5A-core interaction, disturbance of the subcellular localization of NS5A, and disruption of virion production. Replacing the same serine cluster with glutamic acid, which mimics the presence of phosphoserines, partially preserved the NS5A-core interaction and virion production, suggesting that phosphorylation of these serine residues is important for virion production. In addition, we found that the alanine substitutions in the serine cluster suppressed the association of the core protein with viral genome RNA, possibly resulting in the inhibition of nucleocapsid assembly. These results suggest that NS5A plays a key role in regulating the early phase of HCV particle formation by interacting with core protein and that its C-terminal serine cluster is a determinant of the NS5A-core interaction.

3.1128 Lysyl Oxidase Oxidizes Cell Membrane Proteins and Enhances the Chemotactic Response of Vascular Smooth Muscle Cells
Lucero, H.A. et al
J. Biol. Chem., ***283(35)***, *24103-24117 (2008)*

Lysyl oxidase (LOX) is a potent chemokine inducing the migration of varied cell types. Here we demonstrate that inhibition of LOX activity by β-aminopropionitrile (BAPN) in cultured rat aortic smooth muscle cells (SMCs) reduced the chemotactic response and sensitivity of these cells toward LOX and toward PDGF-BB. The chemotactic activity of PDGF-BB was significantly enhanced in the presence of a non-chemotactic concentration of LOX. We considered the possibility that extracellular LOX may oxidize cell surface proteins, including the PDGF receptor-β (PDGFR-β), to affect PDGF-BB-induced chemotaxis. Plasma membranes purified from control SMC contained oxidized PDGFR-β. The oxidation of this receptor and other membrane proteins was largely prevented in cells preincubated with BAPN. Addition of purified LOX to these cells restored the profile of oxidized proteins toward that of control cells. The high affinity and capacity for the binding of PDGF-BB by cells containing oxidized PDGFR-β was diminished by ~2-fold when compared with cells in which oxidation by LOX was prevented by BAPN. Phosphorylated members of the PDGFR-β-dependent signal transduction pathway, including PDGFR-β, SHP2, AKT1, and ERK1/ERK2 (p44/42 MAPK), turned over faster in BAPN-treated than in control SMCs. LOX knock-out mouse embryonic fibroblasts mirrored the effect obtained with SMCs treated with BAPN. These novel findings suggest that LOX activity is essential to generate optimal chemotactic sensitivity of cells to chemoattractants by oxidizing specific cell surface proteins, such as PDGFR-β.

3.1129 Intramembrane Processing by Signal Peptide Peptidase Regulates the Membrane Localization of Hepatitis C Virus Core Protein and Viral Propagation
Okamoto, K. et al
J. Virol., ***82(17)***, *8349-8361 (2008)*

Hepatitis C virus (HCV) core protein has shown to be localized in the detergent-resistant membrane (DRM), which is distinct from the classical raft fraction including caveolin, although the biological significance of the DRM localization of the core protein has not been determined. The HCV core protein is cleaved off from a precursor polyprotein at the lumen side of Ala^{191} by signal peptidase and is then further processed by signal peptide peptidase (SPP) within the transmembrane region. In this study, we examined the role of SPP in the localization of the HCV core protein in the DRM and in viral propagation. The C terminus of the HCV core protein cleaved by SPP in 293T cells was identified as Phe^{177} by mass spectrometry. Mutations introduced into two residues (Ile^{176} and Phe^{177}) upstream of the cleavage site of

the core protein abrogated processing by SPP and localization in the DRM fraction. Expression of a dominant-negative SPP or treatment with an SPP inhibitor, L685,458, resulted in reductions in the levels of processed core protein localized in the DRM fraction. The production of HCV RNA in cells persistently infected with strain JFH-1 was impaired by treatment with the SPP inhibitor. Furthermore, mutant JFH-1 viruses bearing SPP-resistant mutations in the core protein failed to propagate in a permissive cell line. These results suggest that intramembrane processing of HCV core protein by SPP is required for the localization of the HCV core protein in the DRM and for viral propagation.

3.1130 Ral-regulated interaction between Sec5 and paxillin targets Exocyst to focal complexes during cell migration

Spiczka, K.S. and Yeaman, C.
J. Cell Sci., ***121***, *2880-2891 (2008)*

Changes in cellular behavior that cause epithelial cells to lose adhesiveness, acquire a motile invasive phenotype and metastasize to secondary sites are complex and poorly understood. Molecules that normally function to integrate adhesive spatial information with cytoskeleton dynamics and membrane trafficking probably serve important functions in cellular transformation. One such complex is the Exocyst, which is essential for targeted delivery of membrane and secretory proteins to specific plasma membrane sites to maintain epithelial cell polarity. Upon loss of cadherin-mediated adhesion in Dunning R3327-5'A prostate tumor cells, Exocyst localization shifts from lateral membranes to tips of protrusive membrane extensions. Here, it colocalizes and co-purifies with focal complex proteins that regulate membrane trafficking and cytoskeleton dynamics. These sites are the preferred destination of post-Golgi transport vesicles ferrying biosynthetic cargo, such as α_5-integrin, which mediates adhesion of cells to the substratum, a process essential to cell motility. Interference with Exocyst activity impairs integrin delivery to plasma membrane and inhibits tumor cell motility and matrix invasiveness. Localization of Exocyst and, by extension, targeting of Exocyst-dependent cargo, is dependent on Ral GTPases, which control association between Sec5 and paxillin. Overexpression of Ral-uncoupled Sec5 mutants inhibited Exocyst interaction with paxillin in 5'A cells, as did RNAi-mediated reduction of either RalA or RalB. Reduction of neither GTPase significantly altered steady-state levels of assembled Exocyst in these cells, but did change the observed localization of Exocyst proteins.

3.1131 Multistep, sequential control of the trafficking and function of the multiple sulfatase deficiency gene product, SUMF1 by PDI, ERGIC-53 and ERp44

Fraldi, A. et al
Human Mol. Genet., ***17(17)***, *2610-2621 (2008)*

Sulfatase modifying factor 1 (SUMF1) encodes for the formylglicine generating enzyme, which activates sulfatases by modifying a key cysteine residue within their catalytic domains. SUMF1 is mutated in patients affected by multiple sulfatase deficiency, a rare recessive disorder in which all sulfatase activities are impaired. Despite the absence of canonical retention/retrieval signals, SUMF1 is largely retained in the endoplasmic reticulum (ER), where it exerts its enzymatic activity on nascent sulfatases. Part of SUMF1 is secreted and paracrinally taken up by distant cells. Here we show that SUMF1 interacts with protein disulfide isomerase (PDI) and ERp44, two thioredoxin family members residing in the early secretory pathway, and with ERGIC-53, a lectin that shuttles between the ER and the Golgi. Functional assays reveal that these interactions are crucial for controlling SUMF1 traffic and function. PDI couples SUMF1 retention and activation in the ER. ERGIC-53 and ERp44 act downstream, favoring SUMF1 export from and retrieval to the ER, respectively. Silencing ERGIC-53 causes proteasomal degradation of SUMF1, while down-regulating ERp44 promotes its secretion. When over-expressed, each of three interactors favors intracellular accumulation. Our results reveal a multistep control of SUMF1 trafficking, with sequential interactions dynamically determining ER localization, activity and secretion.

3.1132 Pmp-Like Proteins Pls1 and Pls2 Are Secreted into the Lumen of the Chlamydia trachomatis Inclusion

Jorgensen, I. and Valdivia, R.H.
Infect. Immun., ***76(9)***, *3940-3950 (2008)*

The obligate intracellular pathogen *Chlamydia trachomatis* secretes effector proteins across the membrane of the pathogen-containing vacuole (inclusion) to modulate host cellular functions. In an immunological screen for secreted chlamydial proteins, we identified CT049 and CT050 as potential inclusion membrane-associated proteins. These acidic, nonglobular proteins are paralogously related to the passenger domain of

the polymorphic membrane protein PmpC and, like other Pmp proteins, are highly polymorphic among *C. trachomatis* ocular and urogenital strains. We generated antibodies to these Pmp-like secreted (Pls) proteins and determined by immunofluorescence microscopy that Pls1 (CT049) and Pls2 (CT050) localized to globular structures within the inclusion lumen and at the inclusion membrane. Fractionation of membranes and cytoplasmic components from infected cells by differential and density gradient centrifugation further indicated that Pls1 and Pls2 associated with membranes distinct from the bulk of bacterial and inclusion membranes. The accumulation of Pls1 and, to a lesser extent, Pls2 in the inclusion lumen was insensitive to the type III secretion inhibitor C1, suggesting that this translocation system is not essential for Pls protein secretion. In contrast, Pls secretion and stability were sensitive to low levels of β-lactam antibiotics, suggesting that a functional cell wall is required for Pls secretion from the bacterial cell. Finally, we tested the requirement for these proteins in *Chlamydia* infection by microinjecting anti-Pls1 and anti-Pls2 antibodies into infected cells. Coinjection of anti-Pls1 and -Pls2 antibodies partially inhibited expansion of the inclusion. Because Pls proteins lack classical *sec*-dependent secretion signals, we propose that Pls proteins are secreted into the inclusion lumen by a novel mechanism to regulate events important for chlamydial replication and inclusion expansion.

3.1133 Subcellular distribution of APP/C99 and amyloid-beta peptide is altered in CHO NPC1-null cells compared to CHOwt

Posavec, M., Boskovic, D., Goate, A., Hecimovic, S. and Boskovic, R.
*Alzheimer's and Dementia, **4(4)**, Suppl. 1, T353 (2008)*

Background: A sphingolipid storage disease (SLSD) Niemann Pick type C (NPC) is caused by dysfunction of NPC1 protein which leads to accumulation of free cholesterol and glycosphingolipids in endosomal/lysosomal compartments. It has been recently shown that this defect leads to increased formation of amyloid-beta (Abeta) peptide, and is accompanied by altered localization of presenilin 1 to early/late endosomes. We hypothesized that cholesterol accumulation upon NPC1 loss of function leads to increased APP/C99 localization in endosomes and increased formation of Abeta in these compartments. To test this we monitored subcellular localization of APP/C99 and Abeta between CHO NPC1-null (M12) and CHOwt cells. Methods: The cells were stably transfected with APPsw-6myc construct. Subcellular distribution of APP processing products and organelle markers was analyzed by subcellular fractionation in an Iodixanol gradient. After centrifugation (20h and 100,000xg), fractions were collected from the top and protein (DC Protein Assay, BioRad) and cholesterol concentrations (AmplexRed cholesterol assay, Molecular Probes) were determined in each fraction. After acetone precipitation, APP processing products and organelle markers were detected by western blotting using specific antibodies, while Abeta levels were determined by ELISA assay (BioSource International, Inc.). Results: We observed that the majority of subcellular markers tested (BIP/GRP78, EEA1 and Rab7) showed an altered subcellular distribution in M12 cells compared to CHOwt, indicating that loss of NPC1 leads to altered membrane/protein trafficking. In addition, markedly increased levels of BIP/GRP78 were detected in M12 vs. CHOwt cells, which has been previously reported in another SLSD - GM1 gangliosidosis. Although APP was found in similar fractions of M12 and CHOwt cells, the proportion of APP in early/late endosomes was higher in M12 cells. In these cells we also observed increased levels of Abeta40 in endosomal fractions. Conclusions: Our results suggest that increased formation of Abeta in CHO NPC1-null cells is due to redistribution of APP/C99 to early/late endosomes. We propose that NPC1 dysfunction leads to increased coupling of presenilin 1 and its substrate C99 in endosome compartments generating increased Abeta.

3.1134 Trafficking of chlamydial antigens to the endoplasmic reticulum of infected epithelial cells

Giles, D.K. and Wyrick, P.B.
*Microbes and Infection, **10**, 1494-1503 (2008)*

Confinement of the obligate intracellular bacterium *Chlamydia trachomatis* to a membrane-bound vacuole, termed an inclusion, within infected epithelial cells neither prevents secretion of chlamydial antigens into the host cytosol nor protects chlamydiae from innate immune detection. However, the details leading to chlamydial antigen presentation are not clear. By immunoelectron microscopy of infected endometrial epithelial cells and in isolated cell secretory compartments, chlamydial major outer membrane protein (MOMP), lipopolysaccharide (LPS) and the inclusion membrane protein A (IncA) were localized to the endoplasmic reticulum (ER) and co-localized with multiple ER markers, but not with markers of the endosomes, lysosomes, Golgi nor mitochondria. Chlamydial LPS was also co-localized with CD1d in the ER. Since the chlamydial antigens, contained in everted inclusion membrane vesicles, were found within the host cell ER, these data raise additional implications for antigen processing by infected uterine epithelial cells for classical and non-classical T cell antigen presentation.

3.1135 The GET Complex Mediates Insertion of Tail-Anchored Proteins into the ER Membrane
Schuldiner, M. et al
*Cell, **134**, 634-645 (2008)*

Tail-anchored (TA) proteins, defined by the presence of a single C-terminal transmembrane domain (TMD), play critical roles throughout the secretory pathway and in mitochondria, yet the machinery responsible for their proper membrane insertion remains poorly characterized. Here we show that Get3, the yeast homolog of the TA-interacting factor Asna1/Trc40, specifically recognizes TMDs of TA proteins destined for the secretory pathway. Get3 recognition represents a key decision step, whose loss can lead to misinsertion of TA proteins into mitochondria. Get3-TA protein complexes are recruited for endoplasmic reticulum (ER) membrane insertion by the Get1/Get2 receptor. In vivo, the absence of Get1/Get2 leads to cytosolic aggregation of Get3-TA complexes and broad defects in TA protein biogenesis. In vitro reconstitution demonstrates that the Get proteins directly mediate insertion of newly synthesized TA proteins into ER membranes. Thus, the GET complex represents a critical mechanism for ensuring efficient and accurate targeting of TA proteins.

3.1136 Sulfotransferase 2B1b in human breast: Differences in subcellular localization in African American and Caucasian women
Dumas, N.A., He, D., Frost, A.R. and Falany, C.N.
*J. Steroid Biochem Mol. Biol., **111**, 171-177 (2008)*

Breast cancer (BC) is the most commonly diagnosed cancer among American women; however, the development of post-menopausal BC is significantly lower in African Americans as compared to Caucasians. Hormonal stimulation is important in BC development and differences in the conversion of dehydroepiandrosterone (DHEA) into estrogens may be involved in the lower incidence of post-menopausal BC in African American women. DHEA sulfation by sulfotransferase 2B1b (SULT2B1b) is important in regulating the conversion of DHEA into estrogens in tissues. SULT2B1b is localized in both cytosol and nuclei of some tissues including cancerous and associated-normal breast tissue. Immunohistochemical staining was used to evaluate the total expression and subcellular localization of SULT2B1b in African American and Caucasian breast tissues. Cell fractionation, immunoblot analysis and sulfation assays were used to characterize the subcellular expression and activity of SULT2B1b in BC tissues and T-47D breast adenocarcinoma cells. Immunohistochemical analysis of SULT2B1b showed that African Americans had a significantly greater amount of SULT2B1b in epithelial cells of associated-normal breast tissue as compared to Caucasians. Also, more SULT2B1b in African American associated-normal breast epithelial cells was localized in the nuclei than in Caucasians. Equivalent levels of SULT2B1b were detected in breast adenocarcinoma tissues from both African American and Caucasian women. Nuclei isolation and immunoblot analysis of both BC tissue and human T-47D breast adenocarcinoma cells demonstrated that SULT2B1b is present in nuclei and cytoplasm.

3.1137 Distinct binding sites for the ATPase and substrate-binding domain of human Hsp70 on the cell surface of antigen presenting cells
Zitzler, S., Hellwig, A., Hartl, F-U., Wieland, F. And Diestelkötter-Bachert, P.
*Mol. Immunol., **45**, 3974-3983 (2008)*

Hsp70 has high potential as an immune-adjuvant molecule: it mediates cytokine expression and maturation of antigen presenting cells (APCs) and also elicits a cytotoxic T-lymphocyte (CTL) response to antigenic peptides. How Hsp70 interacts with APCs is only poorly understood. Various surface proteins have been implicated in binding Hsp70 but their role in antigen presentation has remained controversial.
The specific aim of this work was to determine the binding and uptake of human full-length Hsp70 as well as its separate ATPase (N70) and substrate-binding domains (C70) by APCs. Using laser scanning microscopy and FACS analysis, we established the existence of at least two distinct receptors for Hsp70, which are localized to distinct microdomains of the APC membrane. These receptors interact with the N70 and C70 domains of Hsp70, respectively. This observation was supported by the finding of a substantial portion of Hsp70 and C70, but not N70, in a detergent resistant membrane fraction. Accordingly, C70 and N70 did not compete with each other for binding. The bound proteins were rapidly internalized, with N70 and C70 localizing to separate endosomal compartments. Similarly, internalized free and peptide-loaded Hsp70 segregated rapidly within the cell. Efficient cross presentation of antigenic peptide bound to Hsp70 or C70 was demonstrated with the B3Z read out system. Consequently, the interaction of C70 with its putative receptor seems to be responsible for Hsp70-mediated cross presentation. Future studies should

make use of C70 in identifying the uptake receptor of Hsp70–peptide complexes. In addition we could observe a stimulation of uptake of free peptide by preincubation with Hsp70 and N70, but not C70, whereas an Hsp-dependent cytokine secretion could not be detected. Consequently, by employing the individual domains it may be possible to distinguish between the different outcomes of Hsp70 treatment, like immune stimulation, DC maturation and antigen-specific responses.

3.1138 The insulin-regulated aminopeptidase IRAP is colocalised with GLUT4 in the mouse hippocampus - potential role in modulation of glucose uptake in neurones?
Fernando, R.N., Albiston, A.L. and Chai, S.Y.
Eur. J. Neurosci., ***28***, *588-598 (2008)*

It is proposed that insulin-regulated aminopeptidase (IRAP) is the site of action of two peptides, angiotensin IV and LVV-hemorphin 7, which have facilitatory effects on learning and memory. In fat and muscles, IRAP codistributes with the insulin-responsive glucose transporter GLUT4 in specialised vesicles, where it plays a role in the tethering and/or trafficking of these vesicles. This study investigated whether an analogous system exists in two functionally distinct regions of the brain, the hippocampus and the cerebellum. In the hippocampus, IRAP was found in the pyramidal neurones where it exhibited a high degree of colocalisation with GLUT4. Consistent with the role of GLUT4 in insulin-responsive tissues, the glucose transporter was thought to be responsible for facilitating glucose uptake into these pyramidal neurones in response to potassium-induced depolarisation or cAMP activation as the glucose influx was sensitive to indinavir treatment. Angiotensin IV and LVV-hemorphin 7 enhanced this activity-dependent glucose uptake in hippocampal slices. In contrast, in the cerebellum, where the distribution of IRAP was dissociated from GLUT4, the effect of the peptides on glucose uptake was absent. We propose that the modulation of glucose uptake by angiotensin IV and LVV-hemorphin 7 is region-specific and is critically dependent on a high degree of colocalisation between IRAP and GLUT4. These findings also confirm a role for IRAP and GLUT4 in activity-dependent glucose uptake in hippocampal neurones.

3.1139 Separable requirements for cytoplasmic domain of PSGL-1 in leukocyte rolling and signaling under flow
Miner, J.J. et al
Blood, ***112(5)****, 2035-2045 (2008)*

In inflamed venules, leukocytes use P-selectin glycoprotein ligand-1 (PSGL-1) to roll on P-selectin and E-selectin and to activate integrin αLβ2 (lymphocyte function-associated antigen-1, LFA-1) to slow rolling on intercellular adhesion molecule-1 (ICAM-1). Studies in cell lines have suggested that PSGL-1 requires its cytoplasmic domain to localize in membrane domains, to support rolling on P-selectin, and to signal through spleen tyrosine kinase (Syk). We generated "ΔCD" mice that express PSGL-1 without the cytoplasmic domain. Unexpectedly, neutrophils from these mice localized PSGL-1 normally in microvilli, uropods, and lipid rafts. ΔCD neutrophils expressed less PSGL-1 on their surfaces because of inefficient export from the endoplasmic reticulum. Limited digestion of wild-type neutrophils with O-sialoglycoprotein endopeptidase was used to reduce the PSGL-1 density to that on ΔCD neutrophils. At matched PSGL-1 densities, both ΔCD and wild-type neutrophils rolled similarly on P-selectin. However, ΔCD neutrophils rolling on P-selectin did not trigger Syk-dependent activation of LFA-1 to slow rolling on ICAM-1. These data demonstrate that the PSGL-1 cytoplasmic domain is dispensable for leukocyte rolling on P-selectin but is essential to activate β2 integrins to slow rolling on ICAM-1.

3.1140 The presence of an ER exit signal determines the protein sorting upon ER exit in yeast
Watanabe, R., Castillon, G.A., Meury, A. and Riezman, H.
Biochem. J., ***414***, *237-245 (2008)*

In yeast, there are at least two vesicle populations upon ER (endoplasmic reticulum) exit, one containing Gap1p (general aminoacid permease) and a glycosylated α-factor, gpαF (glycosylated proα-factor), and the other containing GPI (glycosylphosphatidylinositol)-anchored proteins, Gas1p (glycophospholipid-anchored surface protein) and Yps1p. We attempted to identify sorting determinants for this protein sorting event in the ER. We found that mutant Gas1 proteins that lack a GPI anchor and/or S/T region (serine- and threonine-rich region), two common characteristic features conserved among yeast GPI-anchored proteins, were still sorted away from Gap1p-containing vesicles. Furthermore, a mutant glycosylated α-factor, gpαGPI, which contains both the GPI anchor and S/T region from Gas1p, still entered Gap1p-containing vesicles, demonstrating that these conserved characteristics do not prevent proteins from entering Gap1p-containing vesicles. gpαF showed severely reduced budding efficiency in the absence of its ER exit

receptor Erv29p, and this residual budding product no longer entered Gap1p-containing vesicles. These results suggest that the interaction of gpαF with Erv29p is essential for sorting into Gap1p-containing vesicles. We compared the detergent solubility of Gas1p and the gpαGPI in the ER with that in ER-derived vesicles. Both GPI-anchored proteins similarly partitioned into the DRM (detergent-resistant membrane) in the ER. Based on the fact that they entered different ER-derived vesicles, we conclude that DRM partitioning of GPI-anchored proteins is not the dominant determinant of protein sorting upon ER exit. Interestingly, upon incorporation into the ER-derived vesicles, gpαGPI was no longer detergent-insoluble, in contrast with the persistent detergent insolubility of Gas1p in the ER-derived vesicles. We present different explanations for the different behaviours of GPI-anchored proteins in distinct ER-derived vesicle populations.

3.1141 Increased basolateral sorting of carcinoembryonic antigen in a polarized colon carcinoma cell line after cholesterol depletion-Implications for treatment of inflammatory bowel disease
Ehehalt, R. et al
World J. Gastroenterol., ***14(10)****, 1528-1533 (2008)*

AIM: To investigate a possible increase of basolateral expression of carcinoembryonic antigen (CEA) by interfering with the apical transport machinery, we studied the effect of cholesterol depletion on CEA sorting and secretion. METHODS: Cholesterol depletion was performed in polarized Caco-2 cells using lovastatin and methyl-beta-cyclodextrin. RESULTS: We show that CEA is predominantly expressed and secreted at the apical surface. Reduction of the cholesterol level of the cell by 40%-50% with lovastatin and methyl-beta-cyclodextrin led to a significant change of the apical-to-basolateral transport ratio towards the basolateral membrane. CONCLUSION: As basolateral expression of CEA has been suggested to have anti-inflamatory properties, Cholesterol depletion of enterocytes might be a potential approach to influence the course of inflammatory bowel disease.

3.1142 The Viral Oncoprotein LMP1 Exploits TRADD for Signaling by Masking Its Apoptotic Activity
Schneider, F. et al
PloS Biology, ***6(1)****, 86-98 (2008)*

The tumor necrosis factor (TNF)-receptor 1–associated death domain protein (TRADD) mediates induction of apoptosis as well as activation of NF-κB by cellular TNF-receptor 1 (TNFR1). TRADD is also recruited by the latent membrane protein 1 (LMP1) oncoprotein of Epstein-Barr virus, but its role in LMP1 signaling has remained enigmatic. In human B lymphocytes, we have generated, to our knowledge, the first genetic knockout of *TRADD* to investigate TRADD's role in LMP1 signal transduction. Our data from TRADD-deficient cells demonstrate that TRADD is a critical signaling mediator of LMP1 that is required for LMP1 to recruit and activate I-κB kinase β (IKKβ). However, in contrast to TNFR1, LMP1-induced TRADD signaling does not induce apoptosis. Searching for the molecular basis for this observation, we characterized the 16 C-terminal amino acids of LMP1 as an autonomous and unique virus-derived TRADD-binding domain. Replacing the death domain of TNFR1 by LMP1′s TRADD-binding domain converts TNFR1 into a nonapoptotic receptor that activates NF-κB through a TRAF6-dependent pathway, like LMP1 but unlike wild-type TNFR1. Thus, the unique interaction of LMP1 with TRADD encodes the transforming phenotype of viral TRADD signaling and masks TRADD's pro-apoptotic function.

3.1143 Vesicular Egress of Non-Enveloped Lytic Parvoviruses Depends on Gelsolin Functioning
Bär, S., Daeffler, L., Rommelaere, J. And Nüesch, J.P.F.
PloS Pathogens, ***4(8)****, 1-11 (2008)*

The autonomous parvovirus Minute Virus of Mice (MVM) induces specific changes in the cytoskeleton filaments of infected permissive cells, causing in particular the degradation of actin fibers and the generation of “actin patches.” This is attributed to a virus-induced imbalance between the polymerization factor N-WASP (Wiscott-Aldrich syndrome protein) and gelsolin, a multifunctional protein cleaving actin filaments. Here, the focus is on the involvement of gelsolin in parvovirus propagation and virus-induced actin processing. Gelsolin activity was knocked-down, and consequences thereof were determined for virus replication and egress and for actin network integrity. Though not required for virus replication or progeny particle assembly, gelsolin was found to control MVM (and related H1-PV) transport from the nucleus to the cell periphery and release into the culture medium. Gelsolin-dependent actin degradation and progeny virus release were both controlled by (NS1)/CKIIα, a recently identified complex between a cellular protein kinase and a MVM non-structural protein. Furthermore, the export of newly synthesized

virions through the cytoplasm appeared to be mediated by (virus-modified) lysomal/late endosomal vesicles. By showing that MVM release, like entry, is guided by the cytoskeleton and mediated by vesicles, these results challenge the current view that egress of non-enveloped lytic viruses is a passive process.

3.1144 Endophilin B1 as a Novel Regulator of Nerve Growth Factor/ TrkA Trafficking and Neurite Outgrowth

Wan, J. et al

J. Neurosci., ***28(36)****, 9002-9012 (2008)*

Neurotrophins and their cognate receptors Trks are important regulators of neuronal survival and differentiation. Recent studies reveal that internalization and trafficking of Trks play a critical role in neurotrophin-mediated signaling. At present, little is known of the molecular events that mediate this process. In the current study, we show that endophilin B1 is a novel regulator of nerve growth factor (NGF) trafficking. We found that endophilin B1 interacts with both TrkA and early endosome marker EEA1. Interestingly, knockdown of endophilin B1 results in enlarged EEA1-positive vesicles in NGF-treated PC12 cells. This is accompanied by increased lysosomal targeting of NGF/TrkA and TrkA degradation, and reduced total TrkA levels. In addition, knockdown of endophilin B1 attenuates Erk1/2 activation in the endosomal fraction after NGF treatment. This is accompanied by a marked inhibition of NGF-induced gene transcription and neurite outgrowth in endophilin B1-knocked down cells. Our observations implicate endophilin B1 as a novel regulator of NGF trafficking, thereby affecting TrkA levels and downstream signaling on endosomes to mediate biological functions of NGF.

3.1145 Conditioning the heart induces formation of signalosomes that interact with mitochondria to open mitoKATP channels

Quinlan, C.L. et al

Am. J. Physiol. Heart Circ. Physiol., ***295****, H953-H961 (2008)*

Perfusion of the heart with bradykinin triggers cellular signaling events that ultimately cause opening of mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channels, increased H_2O_2 production, inhibition of the mitochondrial permeability transition (MPT), and cardioprotection. We hypothesized that the interaction of bradykinin with its receptor induces the assembly of a caveolar signaling platform (signalosome) that contains the enzymes of the signaling pathway and that migrates to mitochondria to induce mitoK_{ATP} channel opening. We developed a novel method for isolating and purifying signalosomes from Langendorff-perfused rat hearts treated with bradykinin. Fractions containing the signalosomes were found to open mitoK_{ATP} channels in mitochondria isolated from untreated hearts via the activation of mitochondrial PKC-ε. mitoK_{ATP} channel opening required signalosome-dependent phosphorylation of an outer membrane protein. Immunodetection analysis revealed the presence of the bradykinin B_2 receptor only in the fraction isolated from bradykinin-treated hearts. Immunodetection and immunogold labeling of caveolin-3, as well as sensitivity to cholesterol depletion and resistance to Triton X-100, attested to the caveolar nature of the signalosomes. Ischemic preconditioning, ischemic postconditioning, and perfusion with ouabain also led to active signalosome fractions that opened mitoK_{ATP} channels in mitochondria from untreated hearts. These results provide initial support for a novel mechanism for signal transmission from a plasma membrane receptor to mitoK_{ATP} channels.

3.1146 Use of Fluorescence-activated Vesicle Sorting for Isolation of Naked2-associated, Basolaterally Targeted Exocytic Vesicles for Proteomics Analysis

Cao, Z. et al

Mol. Cell. Proteomics, ***7(9)****, 1651-1667 (2008)*

By interacting with the cytoplasmic tail of a Golgi-processed form of transforming growth factor-α (TGF α), Naked2 coats TGFα-containing exocytic vesicles and directs them to the basolateral corner of polarized epithelial cells where the vesicles dock and fuse in a Naked2 myristoylation-dependent manner. These TGFα-containing Naked2-associated vesicles are not directed to the subapical Sec6/8 exocyst complex as has been reported for other basolateral cargo, and thus they appear to represent a distinct set of basolaterally targeted vesicles. To identify constituents of these vesicles, we exploited our finding that myristoylation-deficient Naked2 G2A vesicles are unable to fuse at the plasma membrane. Isolation of a population of myristoylation-deficient, green fluorescent protein-tagged G2A Naked2-associated vesicles was achieved by biochemical enrichment followed by flow cytometric fluorescence-activated vesicle sorting. The protein content of these plasma membrane de-enriched, flow-sorted fluorescent G2A Naked2 vesicles was determined by LC/LC-MS/MS analysis. Three independent isolations were performed, and

389 proteins were found in all three sets of G2A Naked2 vesicles. Rab10 and myosin IIA were identified as core machinery, and Na^+/K^+-ATPase α1 was identified as an additional cargo within these vesicles. As an initial validation step, we confirmed their presence and that of three additional proteins tested (annexin A1, annexin A2, and IQGAP1) in wild-type Naked2 vesicles. To our knowledge, this is the first large scale protein characterization of a population of basolaterally targeted exocytic vesicles and supports the use of fluorescence-activated vesicle sorting as a useful tool for isolation of cellular organelles for comprehensive proteomics analysis.

3.1147 Phosphorylation, lipid raft interaction and traffic of α-synuclein in a yeast model for Parkinson
Zabrocki, P. et al
Biochim. Biophys. Acta., ***1783(10)***, *1767-1780 (2008)*

Parkinson's disease is a neurodegenerative disorder characterized by the formation of Lewy bodies containing aggregated α-synuclein. We used a yeast model to screen for deletion mutants with mislocalization and enhanced inclusion formation of α-synuclein. Many of the mutants were affected in functions related to vesicular traffic but especially mutants in endocytosis and vacuolar degradation combined inclusion formation with enhanced α-synuclein-mediated toxicity. The screening also allowed for identification of casein kinases responsible for α-synuclein phosphorylation at the plasma membrane as well as transacetylases that modulate the α-synuclein membrane interaction. In addition, α-synuclein was found to associate with lipid rafts, a phenomenon dependent on the ergosterol content. Together, our data suggest that toxicity of α-synuclein in yeast is at least in part associated with endocytosis of the protein, vesicular recycling back to the plasma membrane and vacuolar fusion defects, each contributing to the obstruction of different vesicular trafficking routes.

3.1148 Isolation and characterization of mutant animal cell line defective in alkyl-dihydroxyacetonephosphate synthase: Localization and transport of plasmalogens to post-Golgi compartments
Honsho, M., Yagita, Y., Kinoshita, N. and Fujuki, Y.
Biochim. Biophys. Acta, ***1783(10)***, *1857-1865 (2008)*

We herein isolated plasmalogen-deficient Chinese hamster ovary (CHO) mutant, ZPEG251, with a phenotype of normal import of peroxisomal matrix and membrane proteins. In ZPEG251, plasmenylethanolamine (PlsEtn) was severely reduced. Complementation analysis by expression of genes responsible for the plasmalogen biogenesis suggested that alkyl-dihydroxyacetonephosphate synthase (ADAPS), catalyzing the second step of plasmalogen biogenesis, was deficient in ZPEG251. ADAPS mRNA was barely detectable as verified by Northern blot and reverse transcription-PCR analyses. Defect of ADAPS expression was also assessed by immunoblot. As a step toward delineating functional roles of PlsEtn, we investigated its subcellular localization. PlsEtn was localized to post-Golgi compartments and enriched in detergent-resistant membranes. Transport of PlsEtn to post-Golgi compartments was apparently affected by lowering cellular ATP, but not by inhibitors of microtubule assembly and vesicular transport. Partitioning of cholesterol and sphingomyelin, a typical feature of lipid rafts, was not impaired in plasmalogen-deficient cells, including peroxisome assembly-defective mutants, hence suggesting that PlsEtn was not essential for lipid-raft architecture in CHO cells.

3.1149 Occludin oligomeric assembly at tight junctions of the blood-brain barrier is disrupted by peripheral inflammatory hyperalgesia
McCaffrey, G. et al
J. Neurochem., ***106***, *2395-2409 (2008)*

Tight junctions (TJs) at the blood-brain barrier (BBB) dynamically alter paracellular diffusion of blood-borne substances from the peripheral circulation to the CNS in response to external stressors, such as pain, inflammation, and hypoxia. In this study, we investigated the effect of λ-carrageenan-induced peripheral inflammatory pain (i.e., hyperalgesia) on the oligomeric assembly of the key TJ transmembrane protein, occludin. Oligomerization of integral membrane proteins is a critical step in TJ complex assembly that enables the generation of tightly packed, large multiprotein complexes capable of physically obliterating the interendothelial space to inhibit paracellular diffusion. Intact microvessels isolated from rat brains were fractionated by detergent-free density gradient centrifugation, and gradient fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/ Western blot. Injection of λ-carrageenan into the rat hind paw produced after 3 h a marked change in the relative amounts of oligomeric, dimeric, and monomeric occludin isoforms associated with different plasma membrane lipid raft domains and

intracellular compartments in endothelial cells at the BBB. Our findings suggest that increased BBB permeability (i.e., leak) associated with λ-carrageenan-induced peripheral inflammatory pain is promoted by the disruption of disulfide-bonded occludin oligomeric assemblies, which renders them incapable of forming an impermeant physical barrier to paracellular transport.

3.1150 Cathepsin D Is the Main Lysosomal Enzyme Involved in the Degradation of α-Synuclein and Generation of Its Carboxy-Terminally Truncated Species
Sevlever, D., Jiang, P. and Yen, S-H.C.
*Biochemistry, **47**, 9678-9687 (2008)*

α-Synuclein is likely to play a key role in the development of Parkinson's disease as well as other synucleinopathies. In animal models, overexpression of full-length or carboxy-terminally truncated α-synuclein has been shown to produce pathology. Although the proteosome and lysosome have been proposed to play a role in the degradation of α-synuclein, the enzyme(s) involved in α-synuclein clearance and generation of its carboxy-terminally truncated species have not been identified. In this study, the role of cathepsin D and calpain I in these processes was analyzed. *In vitro* experiments, using either recombinant or endogenous α-synuclein as substrates and purified cathepsin D or lysosomes, demonstrated that cathepsin D degraded α-synuclein very efficiently, and that limited proteolysis resulted in the generation of carboxy-terminally truncated species. Purified calpain I also cleaved α-synuclein, but carboxy-terminally truncated species were not the main cleavage products, and calpain I activity present in cellular lysates was not able to degrade the protein. Knockdown of cathepsin D in cells overexpressing wild-type α-synuclein increased total α-synuclein levels by 28% and lysosomal α-synuclein by 2-fold. In *in vitro* experiments, pepstatin A completely blocked the degradation of α-synuclein in purified lysosomes. Furthermore, lysosomes isolated from cathepsin D knockdown cells showed a marked reduction in α-synuclein degrading activity, indicating that cathepsin D is the main lysosomal enzyme involved in α-synuclein degradation. Our findings suggest that upregulation of cathepsin D could be an additional therapeutic strategy to lessen α-synuclein burden in synucleinopathies.

3.1151 Discrimination between exosomes and HIV-1: Purification of both vesicles from cell-free supernatants
Cantin, R., Diou, J., Belanger, D., Tremblay, A.M. and Gilbert, C.
*J. Immunol. Methods, **338**, 21-30 (2008)*

Although enveloped retroviruses bud from the cell surface of T lymphocytes, they use the endocytic pathway and the internal membrane of multivesicular bodies for their assembly and release from macrophages and dendritic cells (DCs). Exosomes, physiological nanoparticles produced by hematopoietic cells, egress from this same pathway and are similar to retroviruses in terms of size, density, the molecules they incorporate and their ability to activate immune cells. Retroviruses are therefore likely to contaminate *in vitro* preparations of exosomes and vice versa and sucrose gradients are inefficient at separating them. However, we have found that their sedimentation velocities in an iodixanol (Optiprep™) velocity gradient are sufficiently different to allow separation and purification of both vesicles. Using acetylcholinesterase as an exosome marker, we demonstrate that Optiprep™ velocity gradients are very efficient in separating exosomes from HIV-1 particles produced on 293T cells, primary $CD4^+$ T cells, macrophages or DCs, with exosomes collecting at 8.4–12% iodixanol and HIV-1 at 15.6%. We also show that immunodepletion with an anti-acetylcholinesterase antibody rapidly produces highly purified preparations of HIV-1 or exosomes. These findings have applications in fundamental research on exosomes and/or AIDS, as well as in clinical applications where exosomes are involved, more specifically in tumour therapy or in gene therapy using exosomes generated from DCs genetically modified by transfection with virus.

3.1152 Uptake of long chain fatty acids is regulated by dynamic interaction of FAT/CD36 with cholesterol/sphingolipid enriched microdomains (lipid rafts)
Ehehalt, R. et al
*BMC Cell Biol., **9**, 45-56 (2008)*

Background
Mechanisms of long chain fatty acid uptake across the plasma membrane are important targets in treatment of many human diseases like obesity or hepatic steatosis. Long chain fatty acid translocation is achieved by a concert of co-existing mechanisms. These lipids can passively diffuse, but certain membrane proteins can also accelerate the transport. However, we now can provide further evidence that not only proteins but also lipid microdomains play an important part in the regulation of the facilitated uptake process.

Methods
Dynamic association of FAT/CD36 a candidate fatty acid transporter with lipid rafts was analysed by isolation of detergent resistant membranes (DRMs) and by clustering of lipid rafts with antibodies on living cells. Lipid raft integrity was modulated by cholesterol depletion using methyl-β-cyclodextrin and sphingolipid depletion using myriocin and sphingomyelinase. Functional analyses were performed using an [3H]-oleate uptake assay.
Results
Overexpression of FAT/CD36 and FATP4 increased long chain fatty acid uptake. The uptake of long chain fatty acids was cholesterol and sphingolipid dependent. Floating experiments showed that there are two pools of FAT/CD36, one found in DRMs and another outside of these domains. FAT/CD36 co-localized with the lipid raft marker PLAP in antibody-clustered domains at the plasma membrane and segregated away from the non-raft marker GFP-TMD. Antibody cross-linking increased DRM association of FAT/CD36 and accelerated the overall fatty acid uptake in a cholesterol dependent manner. Another candidate transporter, FATP4, was neither present in DRMs nor co-localized with FAT/CD36 at the plasma membrane.
Conclusion
Our observations suggest the existence of two pools of FAT/CD36 within cellular membranes. As increased raft association of FAT/CD36 leads to an increased fatty acid uptake, dynamic association of FAT/CD36 with lipid rafts might regulate the process. There is no direct interaction of FATP4 with lipid rafts or raft associated FAT/CD36. Thus, lipid rafts have to be considered as targets for the treatment of lipid disorders.

3.1153 The unique architecture of Bunyamwera virus factories around the Golgi complex
Fontana, J., Lopez-Montero, N., Elliott, R.M., Fernandez, J.J. and Risco, C.
Cell. Microbiol., ***10(10)****, 2112-2028 (2008)*

Viral factories are novel structures built by viruses in infected cells. During their construction organelles are recruited and build a large scaffold for viral replication and morphogenesis. We have studied how a bunyavirus uses the Golgi to build the factory. With the help of confocal and 3D ultrastructural imaging together with molecular mapping *in situ* and *in vitro* we have characterized a tubular structure that harbours the viral replication complexes in a globular domain. Numerous ribonucleoproteins were released from purified tubes disrupted *in vitro*. Actin and myosin I were identified by peptide mass fingerprinting in isolated tubes while actin and the viral NSm non-structural protein were detected in the tubes' internal proteinaceous scaffold by immunogold labelling. Studies with NSm deletion mutants and drugs affecting actin showed that both NSm and actin are key factors for tube and virus assembly in Golgi. Three-dimensional reconstructions based on oriented serial sections of infected cells showed that tubes anchor cell organelles to Golgi stacks and make contacts with intracellular viruses. We propose that this new structure, unique among enveloped viruses, assembles in association with the most stable component of Golgi stacks, the actin-containing matrix scaffold, connecting viral replication and morphogenesis inside viral factories.

3.1154 Absence of 2-Hydroxylated Sphingolipids Is Compatible with Normal Neural Development But Causes Late-Onset Axon and Myelin Sheath Degeneration
Zöller, I. et al
J. Neurosci., ***28(39)****, 9741-9754 (2008)*

Sphingolipids containing 2-hydroxylated fatty acids are among the most abundant lipid components of the myelin sheath and therefore are thought to play an important role in formation and function of myelin. To prove this hypothesis, we generated mice lacking a functional fatty acid 2-hydroxylase (FA2H) gene. FA2H-deficient ($FA2H^{-/-}$) mice lacked 2-hydroxylated sphingolipids in the brain and in peripheral nerves. In contrast, nonhydroxylated galactosylceramide was increased in $FA2H^{-/-}$ mice. However, oligodendrocyte differentiation examined by *in situ* hybridization with cRNA probes for proteolipid protein and PDGFα receptor and the time course of myelin formation were not altered in $FA2H^{-/-}$ mice compared with wild-type littermates. Nerve conduction velocity measurements of sciatic nerves revealed no significant differences between $FA2H^{-/-}$ and wild-type mice. Moreover, myelin of $FA2H^{-/-}$ mice up to 5 months of age appeared normal at the ultrastructural level, in the CNS and peripheral nervous system. Myelin thickness and g-ratios were normal in $FA2H^{-/-}$ mice. Aged (18-month-old) $FA2H^{-/-}$ mice, however, exhibited scattered axonal and myelin sheath degeneration in the spinal cord and an even more pronounced loss of stainability of myelin sheaths in sciatic nerves. These results show that structurally and functionally

normal myelin can be formed in the absence of 2-hydroxylated sphingolipids but that its long-term maintenance is strikingly impaired. Because axon degeneration appear to start rather early with respect to myelin degenerations, these lipids might be required for glial support of axon function.

3.1155 Kinesin KIF4 Regulates Intracellular Trafficking and Stability of the Human Immunodeficiency Virus Type 1 Gag Polyprotein
Martinez, N., Xue, X., Berro, R.G., Kreitzer, G. and Resh, M.D.
J. Virol., ***82(20)***, *9937-9950 (2008)*

Retroviral Gag proteins are synthesized as soluble, myristoylated precursors that traffic to the plasma membrane and promote viral particle production. The intracellular transport of human immunodeficiency virus type 1 (HIV-1) Gag to the plasma membrane remains poorly understood, and cellular motor proteins responsible for Gag movement are not known. Here we show that disrupting the function of KIF4, a kinesin family member, slowed temporal progression of Gag through its trafficking intermediates and inhibited virus-like particle production. Knockdown of KIF4 also led to increased Gag degradation, resulting in reduced intracellular Gag protein levels; this phenotype was rescued by reintroduction of KIF4. When KIF4 function was blocked, Gag transiently accumulated in discrete, perinuclear, nonendocytic clusters that colocalized with endogenous KIF4, with Ubc9, an E2 SUMO-1 conjugating enzyme, and with SUMO. These studies identify a novel transit station through which Gag traffics en route to particle assembly and highlight the importance of KIF4 in regulating HIV-1 Gag trafficking and stability.

3.1156 Fusion of Enhanced Green Fluorescent Protein to the Pseudorabies Virus Axonal Sorting Protein Us9 Blocks Anterograde Spread of Infection in Mammalian Neurons
Lyman, M.G:, Curanovic, D., Brideau, A.D. and Enquist, L.W.
J. Virol., ***82(20)***, *10308-10311 (2008)*

Pseudorabies virus encodes a membrane protein (Us9) that is essential for the axonal sorting of virus particles within neurons and anterograde spread in the mammalian nervous system. Enhanced green fluorescent protein (GFP)-tagged Us9 mimicked the trafficking properties of the wild-type protein in nonneuronal cells. We constructed a pseudorabies virus strain that expressed Us9-GFP and tested its spread capabilities in the rat visual system and in primary neuronal cultures. We report that Us9-EGFP does not promote anterograde spread of infection and may disrupt packing of viral membrane proteins in lipid rafts, an essential step for Us9-mediated axonal sorting.

3.1157 An epidermal growth factor (EGF) -dependent interaction between GIT1 and sorting nexin 6 promotes degradation of the EGF receptor
Cavet, M.E., Pang, J., Yin, G. And Berk, B.C.
FASEB J., ***22(10)***, *3607-3616 (2008)*

G-protein coupled receptor (GPCR) kinase-2 interacting protein 1 (GIT1) is a multifunctional scaffolding protein that regulates epidermal growth factor receptor (EGFR) signaling pathways. We demonstrate that GIT1 interacts with sorting nexin 6 (SNX6), a member of the SNX family that increases EGFR trafficking between endosomes and lysosomes, thereby enhancing EGFR degradation. The GIT1-SNX6 interaction is increased 3-fold after treatment with EGF for 60 min. The second coiled-coil domain (CC2; aa 424–474) of GIT1 mediates binding to SNX6. Subcellular fractionation and confocal microscopy data indicate that GIT1 and SNX6 interact in endosomes. Knockdown of GIT1 expression by small interfering RNA decreased the rate of EGF-induced EGFR degradation. Expression of exogenous GIT1 or SNX6 alone did not alter EGFR degradation; however, coexpression of GIT1 and SNX6 decreased EGFR levels both basally and in response to EGF. In contrast, expression of GIT1(CC2 deleted) and SNX6 did not reduce EGFR levels, demonstrating that the interaction between GIT1 and SNX6 was required to regulate EGFR trafficking. Phosphorylation of the EGFR substrate phospholipase C-γ was decreased by coexpression of GIT1 and SNX6. These data demonstrate an endosomal, EGF-regulated interaction between SNX6 and GIT1 that enhances degradation of the EGFR, and thereby alters EGFR signaling. Our findings suggest a new role for GIT1 in tyrosine kinase receptor trafficking.—Cavet, M. E., Pang, J., Yin, G., Berk, B. C. An epidermal growth factor (EGF)-dependent interaction between GIT1 and sorting nexin 6 promotes degradation of the EGF receptor.

3.1158 Formation and function of ceramide-enriched membrane platforms with CD38 during M1-receptor stimulation in bovine coronary arterial myocytes
Jia, S-J. et al

*Am. J. Physiol. Heart Circ. Physiol., **295**, H1743-H1752 (2008)*

CD38 contains an ADP ribosylcyclase domain that mediates intracellular Ca^{2+} signaling by the production of cyclic ADP-ribose (cADPR), but the mechanisms by which the agonists activate this enzyme remain unclear. The present study tested a hypothesis that a special lipid-raft (LR) form, ceramide-enriched lipid platform, contributes to CD38 activation to produce cADPR in response to muscarinic type 1 (M_1) receptor stimulation in bovine coronary arterial myocytes (CAMs). By confocal microscopic analysis, oxotremorine (Oxo), an M_1 receptor agonist, was found to increase LR clustering on the membrane with the formation of a complex of CD38 and LR components such as GM_1, acid sphingomyelinase (ASMase), and ceramide, a typical ceramide-enriched macrodomain. At 80 μM, Oxo increased LR clustering by 78.8%, which was abolished by LR disruptors, methyl-β-cyclodextrin (MCD), or filipin. With the use of a fluorescence resonance energy transfer (FRET) technique, 15.5 ± 1.9% energy transfer rate (vs. 5.3 ± 0.9% of control) between CD38 and LR component, ganglioside M_1 was detected, further confirming the proximity of both molecules. In the presence of MCD or filipin, there were no FRET signals detected. In floated detergent-resistant membrane fractions, CD38 significantly increased in LR fractions of CAMs treated by Oxo. Moreover, MCD or filipin attenuated Oxo-induced production of cADPR via CD38. Functionally, Oxo-induced intracellular Ca^{2+} release and coronary artery constriction via cADPR were also blocked by LR disruption or ASMase inhibition. These results provide the first evidence that the formation of ceramide-enriched lipid macrodomains is crucial for Oxo-induced activation of CD38 to produce cADPR in CAMs, and these lipid macrodomains mediate transmembrane signaling of M_1 receptor activation to produce second messenger cADPR.

3.1159 Determination of the Topology of the Hydrophobic Segment of Mammalian Diacylglycerol Kinase Epsilon in a Cell Membrane and Its Relationship to Predictions from Modeling
Decaffmeyer, M. et al
*J. Mol. Biol., **383**, 797-809 (2008)*

The epsilon isoform of diacylglycerol kinase (DGKε) is unique among mammalian DGKs in having a segment of hydrophobic amino acids comprising approximately residues 20 to 41. Several algorithms predict this segment to be a transmembrane (TM) helix. Using PepLook, we have performed an *in silico* analysis of the conformational preference of the segment in a hydrophobic environment comprising residues 18 to 42 of DGKε. We find that there are two distinct groups of stable conformations, one corresponding to a straight helix that would traverse the membrane and the second corresponding to a bent helix that would enter and leave the same side of the membrane. Furthermore, the calculations predict that substituting the Pro32 residue in the hydrophobic segment with an Ala will cause the hydrophobic segment to favor a TM orientation. We have expressed the P32A mutant of DGKε, with a FLAG tag (an N-terminal 3×FLAG epitope tag) at the amino terminus, in COS-7 cells. We find that this mutation causes a large reduction in both k_{cat} and K_m while maintaining k_{cat}/K_m constant. Specificity of the P32A mutant for substrates with polyunsaturated acyl chains is retained. The P32A mutant also has higher affinity for membranes since it is more difficult to extract from the membrane with high salt concentration or high pH compared with the wild-type DGKε. We also evaluated the topology of the proteins with confocal immunofluorescence microscopy using NIH 3T3 cells. We find that the FLAG tag at the amino terminus of the wild-type enzyme is not reactive with antibodies unless the cell membrane is permeabilized with detergent. We also demonstrate that at least a fraction of the wild-type DGKε is present in the plasma membrane and that comparable amounts of the wild-type and P32A mutant proteins are in the plasma membrane fraction. This indicates that in these cells the hydrophobic segment of the wild-type DGKε is not TM but takes up a bent conformation. In contrast, the FLAG tag at the amino terminus of the P32A mutant is exposed to antibody both before and after membrane permeabilization. This modeling approach thus provides an explanation, not provided by simple predictive algorithms, for the observed topology of this protein in cell membranes. The work also demonstrates that the wild-type DGKε is a monotopic protein.

3.1160 Cripto recruits Furin and PACE4 and controls Nodal trafficking during proteolytic maturation
Blancet, M-H. et al
*EMBO J., **27**, 2580-2591 (2008)*

The glycosylphosphatidylinositol (GPI)-anchored proteoglycan Cripto binds Nodal and its type I receptor Alk4 to activate Smad2,3 transcription factors, but a role during Nodal precursor processing has not been described. We show that Cripto also binds the proprotein convertases Furin and PACE4 and localizes

Nodal processing at the cell surface. When coexpressed as in early embryonic cells, Cripto and uncleaved Nodal already associated during secretion, and a Cripto-interacting region in the Nodal propeptide potentiated the effect of proteolytic maturation on Nodal signalling. Disruption of the trans-Golgi network (TGN) by brefeldin A blocked secretion, but export of Cripto and Nodal to the cell surface was not inhibited, indicating that Nodal is exposed to extracellular convertases before entering the TGN/endosomal system. Density fractionation and antibody uptake experiments showed that Cripto guides the Nodal precursor in detergent-resistant
membranes to endocytic microdomains marked by GFP–Flotillin. We conclude that Nodal processing and endocytosis are coupled in signal-receiving cells.

3.1161 Effects of Monoglycerides on P-Glycoprotein: Modulation of the Activity and Expression in Caco-2 Cell Monolayers

Barta, C.A., Sachs-Barrable, K., Feng, F. and Wasan, K.M.
Mol. Pharmaceut., ***5(5)****, 863-875 (2008)*

The purpose of this study was to analyze the effects of two common monoglyceride components of lipid excipients, 1-monoolein and 1-monostearin, on the activity and expression of P-glycoprotein (P-gp) in Caco-2 cells. Non-cytotoxic concentrations of 1-monoolein and 1-monostearin were determined by assessing membrane permeability and mitochondrial activity in Caco-2 cells, a human colon adenocarcinoma cell line. Concentrations of 500 and 100 μM were used to evaluate P-gp activity through Rh123 accumulation and bifunctional transport studies. The P-gp protein expression levels were quantified through the use of immunoblots. The changes in cell membrane fluidity and nuclear membrane integrity upon the addition of monoglycerides were analyzed by fluorescence anisotropy using DPH and TMA-DPH as the fluorescent labels and by using increasing salt concentrations to release the nuclear contents, respectively. The absorptive flux (apical to basolateral) in the bifunctional transport studies was not found to be statistically significant for the non-cytotoxic concentrations of 1-monoolein and 1-monostearin. However, treatments of 500 and 100 μM of 1-monoolein or 1-monostearin displayed statistically lowered efflux (basolateral to apical, $P < 0.05$) compared to the controls (7.9 ± 0.8, $12.9 \pm 2.6 \times 10^6$ cm/s for 1-monoolein or 11.1 ± 2.0, $11.4 \pm 2.3 \times 10^6$ cm/s for 1-monostearin, respectively, compared to the untreated control, $21.1 \pm 2.9 \times 10^6$ cm/s, $n = 5$). Rh123 accumulation was also found to be enhanced upon 24 h incubation with both concentrations of the monoglycerides; however, only concentrations of 500 μM of the monoglycerides were shown to significantly reduce the P-gp protein expression. The results from this study suggest that these two monoglycerides, common components in various lipid excipients, are inhibitors of P-gp.

3.1162 CDK5-dependent Phosphorylation of the Rho Family GTPase TC10α Regulates Insulin-stimulated GLUT4 Translocation

Okada, S. et al
J. Biol. Chem., ***283(51)****, 35455-35463 (2008)*

Insulin stimulation results in the activation of cyclin-dependent kinase-5 (CDK5) in lipid raft domains via a Fyn-dependent phosphorylation on tyrosine residue 15. In turn, activated CDK5 phosphorylates the Rho family GTP-binding protein TC10α on threonine 197 that is sensitive to the CDK5 inhibitor olomoucine and blocked by small interfering RNA-mediated knockdown of CDK5. The phosphorylation deficient mutant T197A-TC10α was not phosphorylated and excluded from the lipid raft domain, whereas the phosphorylation mimetic mutant (T197D-TC10α) was lipid raft localized. Insulin resulted in the GTP loading of T197D-TC10α but not T197A-TC10α and in parallel, T197D-TC10α but not T197A-TC10α depolymerized cortical actin and inhibited insulin-stimulated GLUT4 translocation. These data demonstrate that CDK5-dependent phosphorylation maintains TC10α in lipid raft compartments thereby disrupting cortical actin, whereas subsequent dephosphorylation of TC10α through inactivation of CDK5 allows for the re-assembly of F-actin. Because cortical actin reorganization is required for insulin-stimulated GLUT4 translocation, these data are consistent with a CDK5-dependent TC10α cycling between lipid raft and non-lipid raft compartments.

3.1163 Bluetongue Virus Outer Capsid Protein VP5 Interacts with Membrane Lipid Rafts via a SNARE Domain

Bhattacharya, B. and Roy, P.
J. Virol., ***82(21)****, 10600-10612 (2008)*

Bluetongue virus (BTV) is a nonenveloped double-stranded RNA virus belonging to the family *Reoviridae*. The two outer capsid proteins, VP2 and VP5, are responsible for virus entry. However, little is known about the roles of these two proteins, particularly VP5, in virus trafficking and assembly. In this study, we used density gradient fractionation and methyl beta cyclodextrin, a cholesterol-sequestering drug, to demonstrate not only that VP5 copurifies with lipid raft domains in both transfected and infected cells, but also that raft domain integrity is required for BTV assembly. Previously, we showed that BTV nonstructural protein 3 (NS3) interacts with VP2 and also with cellular exocytosis and ESCRT pathway proteins, indicating its involvement in virus egress (A. R. Beaton, J. Rodriguez, Y. K. Reddy, and P. Roy, *Proc. Natl. Acad. Sci. USA* 99:13154-13159, 2002; C. Wirblich, B. Bhattacharya, and P. Roy *J. Virol.* **80**:460-473, 2006). Here, we show by pull-down and confocal analysis that NS3 also interacts with VP5. Further, a conserved membrane-docking domain similar to the motif in synaptotagmin, a protein belonging to the SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) family was identified in the VP5 sequence. By site-directed mutagenesis, followed by flotation and confocal analyses, we demonstrated that raft association of VP5 depends on this domain. Together, these results indicate that VP5 possesses an autonomous signal for its membrane targeting and that the interaction of VP5 with membrane-associated NS3 might play an important role in virus assembly.

3.1164 The yeast *O*-acyltransferase Gup1p interferes in lipid metabolism with direct consequences on the sphingolipid-sterol-ordered domains integrity/assembly
Ferreira, C. and Lucas, C.
*Biochim. Biophys. Acta, **1778**, 2648-2653 (2008)*

Saccharomyces cerevisiae Gup1p is a membrane-bound *O*-acyltransferase. Previous works involved *GUP1* in a wide range of crucial processes for cell preservation and functioning. These include cytoskeleton polarization and secretory/endocytic pathway, GPI-anchor remodelling, wall composition and integrity, and membrane lipids, with a reduction in phospholipids and an increase in acylglycerols. DRM fractions were found in considerably lower amounts in *gup1*Δ than in wt strain. Additionally, the proteins presumably associated with lipid micro domains, Gas1p and Pma1p, were present in much smaller amounts in the mutant DRMs. Pma1p is also found in minor quantities in the whole cells extracts of the *gup1*Δ mutant. Accordingly, H^+-ATPase activity was reduced in about 40%. Deletion of *GUP1* resulted in higher sensibility to specific sphingolipid biosynthesis inhibitors and a notorious resistance to ergosterol biosynthesis inhibitors. Furthermore, the majority of mutant cells displayed an even (less punctuated) sterol distribution. The present work presents improvements to DRMs extraction methodology and filipin-sterol staining, provides evidence supporting that Gup1p is involved in lipid metabolism and shows the direct consequences of its absence on the plasma membrane sphingolipid-sterol-ordered domains integrity/assembly.

3.1165 Autocrine activity of tumor-derived membrane vesicles
Hong, B.S., Choi, E.Y., Yoon, Y.J. and Gho, Y.S.
*Eur. J. Cancer Suppl., **6(12)**, 96 (2008)*

Background: Actively growing tumor cells release membrane vesicles into extracellular milieu, and the rate of shedding increases in malignant tumors. Tumor-derived membrane vesicles (TMVs), enriched in most surface antigens and proteases derived from their originating cells, are nowadays gaining attention as important mediators of cell-to-cell communication facilitating processes such as angiogenesis and immune modulation with paracrine functions. Since TMVs have the potential to affect to tumor cells themselves, the role of TMVs in autocrine signals to tumor cells has been investigated.
Material and Methods: We isolated TMVs from SW480, human colorectal adenocarcinoma cells by ultracentrifugation onto sucrose cushion and iodixanol gradients. We characterized TMVs with Western blotting using membrane vesicles markers, density in an iodixanol gradient, and transmission electron microscopy. Cell proliferation was determined by [3H]- thymidine incorporation and cell migration assay was performed in a 48-well microchemotaxis chamber. Intracellular Ca2+ mobilization was determined with the fluorescent Ca2+ indicator fluo-3/AM, and AlexaFluor 488- conjugated phalloidine was used for imaging actin stress fiber formation.
Results: The purified TMVs settled at a density of ~1.110 g/mL, presenting membrane vesicles markers (CD63 and CD81), and almost all TMVs were spherical and bi-layered vesicles ranging from 40 to 150 nm in size. TMVs stimulated proliferation of tumor cells in a dose-dependent manner, with a maximum effect of 2.4±0.2-fold increases upon exposure to 5 mg/ml of TMVs. TMVs increased migration of tumor cells in a dose-dependent manner, with a maximum effect of 4.1±0.9-fold increases upon exposure to 1 mg/ml of TMVs. The treatment of 1 mg/ml of TMVs to fluo-3/AM-loaded tumor cells caused increased intracellular

free Ca2+ release into the cytosol. Moreover, formation of actin stress fibers was enhanced by the treatment of
1 mg/ml of TMVs for 30 min. Theses results suggest that TMVs may deliver autocrine signals to target tumor cells.
Conclusion: We suggest that TMVs may be involved in promoting autocrine signaling to tumor themselves, neighboring or distant tumor cells for the rapid induction of proliferation, migration, as well as survival.
Therefore, modulating biogenesis and functions of TMVs may be potential novel therapeutic strategies for treating pathological states including malignant tumors.

3.1166 Ceramide and raft signaling are linked with each other in UVA radiation-induced gene expression
Grether-Beck, S. et al
*Oncogene, **27**, 4768-4778 (2008)*

Solar ultraviolet A (UVA) (320–400 nm) radiation-induced gene expression in keratinocytes is initiated at the
level of the cell membrane via generation of singlet oxyge and subsequent formation of ceramide from sphingomyelin. We now report that the UVA response also involves raft signaling and that ceramide and raft signaling are linked with each other. Upon UVA irradiation, the lipid composition of rafts decreased 40% in sphingomyelinand 60% in cholesterol (Chol). Also, decrease of Chol increased the susceptibility towards UVA-induced gene expression, whereas increase of Chol completely abolished their capacity to generate signaling ceramides and to mount the subsequent UVA response. This inhibition was not associated with UVA-induced Chol oxidation and was also seen after treatment of cells with plant sterols. The UVA responsiveness depended on the ratio of Chol versus ceramide in rafts. A ratio smaller than 1 permitted
initiation and transduction of the signaling response, whereas a ratio greater than 1, for example, upon sterol
pretreatment, abolished this response, indicating that UVA radiation-induced ceramide signaling is controlled
by the lipid composition of rafts.

3.1167 Involvement of miltefosine-mediated ERK activation in glioma cell apoptosis through Fas regulation
Tewari, R., Sharma, V., Koul, N. and Sen, E.
*J. Neurochem., **107(3)**, 616-627 (2008)*

The anti-neoplastic property of alkyl phospholipids has been tested for the treatment of several malignancies. In this study, we evaluated the efficacy of miltefosine (Hexadecylphosphocholine – an alkyl phospholipids analogue) on glioblastoma multiforme. In this study, we demonstrate that miltefosine-induced apoptosis is accompanied by elevated Fas, Fas-associated death domain (FADD) expression, caspase-8 activity and the increased distribution of Fas and FADD towards lipid raft microdomain to form death inducing signaling complex. Treatment with miltefosine resulted in increase in Ras, extracellular signal-regulated kinase (ERK) and p38MAPK activity. Expression of dominant-negative Ras (Ras N17) attenuated miltefosine-mediated apoptosis. Although inhibition of both ERK and p38MAPK decreased the pro-apoptotic effects of miltefosine, it was the inhibition of ERK and not p38MAPK activation that decreased Fas and FADD expression. An ERK-dependent increase in the expression of γH2AX-involved in response to DNA double-stranded breaks was also observed. Taken together, our findings suggest the involvement of ERK activation in miltefosine-induced glioma cell apoptosis.

3.1168 Delta Protein Kinase C Interacts with the d Subunit of the F1F0 ATPase in Neonatal Cardiac Myocytes Exposed to Hypoxia or Phorbol Ester: IMPLICATIONS FOR F1F0 ATPase REGULATION
Nguyen, T., Ogbi, M. and Johnson, J.A.
*J. Biol. Chem., **283(44)**, 29831-29840 (2008)*

Mitochondrial protein kinase C isozymes have been reported to mediate both cardiac ischemic preconditioning and ischemia/reperfusion injury. In addition, cardiac preconditioning improves the recovery of ATP levels after ischemia/reperfusion injury. We have, therefore, evaluated protein kinase C modulation of the F_1F_0 ATPase in neonatal cardiac myocytes. Exposure of cells to 3 or 100 nM 4β-phorbol 12-myristate-13-acetate induced co-immunoprecipitation of δprotein kinase C (but not α, ϵ, or ζprotein

kinase C) with the d subunit of the F_1F_0 ATPase. This co-immunoprecipitation correlated with 40 ± 3% and 72 ± 9% inhibitions of oligomycin-sensitive F_1F_0 ATPase activity, respectively. We observed prominent expression of δprotein kinase C in cardiac myocyte mitochondria, which was enhanced following a 4-h hypoxia exposure. In contrast, hypoxia decreased mitochondrial ζPKC levels by 85 ± 1%. Following 4 h of hypoxia, F_1F_0 ATPase activity was inhibited by 75 ± 9% and δprotein kinase C co-immunoprecipitated with the d subunit of F_1F_0 ATPase. *In vitro* incubation of protein kinase C with F_1F_0 ATPase enhanced F_1F_0 activity in the absence of protein kinase C activators and inhibited it in the presence of activators. Recombinant δprotein kinase C also inhibited F_1F_0 ATPase activity. Protein kinase C overlay assays revealed δprotein kinase C binding to the d subunit of F_1F_0 ATPase, which was modulated by diacylglycerol, phosphatidylserine, and cardiolipin. Our results suggest a novel regulation of the F_1F_0 ATPase by the δprotein kinase C isozyme.

3.1169 Transport of LDL-derived cholesterol from the NPC1 compartment to the ER involves the trans-Golgi network and the SNARE protein complex
Urano, Y. et al
*PNAS, **105(43)**, 16513-16518 (2008)*

Mammalian cells acquire cholesterol mainly from LDL. LDL enter the endosomes, allowing cholesteryl esters to be hydrolyzed by acid lipase. The hydrolyzed cholesterol (LDL-CHOL) enters the Niemann–Pick type C1 (NPC1)-containing endosomal compartment en route to various destinations. Whether the Golgi is involved in LDL-CHOL transport downstream of the NPC1 compartment has not been demonstrated. Using subcellular fractionation and immunoadsorption to enrich for specific membrane fractions, here we show that, when parental Chinese hamster ovary (CHO) cells are briefly exposed to ^{3}H-cholesteryl linoleate (CL) labeled-LDL, newly liberated ^{3}H-LDL-CHOL appears in membranes rich in trans-Golgi network (TGN) long before it becomes available for re-esterification at the endoplasmic reticulum (ER) or for efflux at the plasma membrane. In mutant cells lacking NPC1, the appearance of newly liberated ^{3}H-LDL-CHOL in the TGN-rich fractions is much reduced. We next report a reconstituted transport system that recapitulates the transport of LDL-CHOL to the TGN and to the ER. The transport system requires ATP and cytosolic factors and depends on functionality of NPC1. We demonstrate that knockdown by RNAi of 3 TGN-specific SNAREs (VAMP4, syntaxin 6, and syntaxin 16) reduces ≥50% of the LDL-CHOL transport in intact cells and in vitro. These results show that vesicular trafficking is involved in transporting a significant portion of LDL-CHOL from the NPC1-containing endosomal compartment to the TGN before its arrival at the ER.

3.1170 Importance of cholesterol-rich membrane microdomains in the interaction of the S protein of SARS-coronavirus with the cellular receptor angiotensin-converting enzyme 2
Glende, J. et al
*Virology, **381**, 215-221 (2008)*

Cholesterol present in the plasma membrane of target cells has been shown to be important for the infection by SARS-CoV. We show that cholesterol depletion by treatment with methyl-β-cyclodextrin (mβCD) affects infection by SARS-CoV to the same extent as infection by vesicular stomatitis virus-based pseudotypes containing the surface glycoprotein S of SARS-CoV (VSV-ΔG-S). Therefore, the role of cholesterol for SARS-CoV infection can be assigned to the S protein and is unaffected by other coronavirus proteins. There have been contradictory reports whether or not angiotensin-converting enzyme 2 (ACE2), the cellular receptor for SARS-CoV, is present in detergent-resistant membrane domains. We found that ACE2 of both Vero E6 and Caco-2 cells co-purifies with marker proteins of detergent-resistant membranes supporting the notion that cholesterol-rich microdomains provide a platform facilitating the efficient interaction of the S protein with the cellular receptor ACE2. To understand the involvement of cholesterol in the initial steps of the viral life cycle, we applied a cell-based binding assay with cells expressing the S protein and cells containing angiotensin-converting enzyme 2 (ACE2). Alternatively, we used a soluble S protein as interaction partner. Depletion of cholesterol from the ACE2-expressing cells reduced the binding of S-expressing cells by 50% whereas the binding of soluble S protein was not affected. This result suggests that optimal infection requires a multivalent interaction between viral attachment protein and cellular receptors.

3.1171 Membrane progestin receptors α and γ in renal epithelium
Lemale, J. et al
*Biochim. Biophys. Acta, **1783**, 2234-2240 (2008)*

Sex hormones have broader effects than regulating reproductive functions. Recent identification of membrane progestin receptors expressed in kidney prompted us to investigate their putative involvement in the renal effects of this hormone. We first focused our investigations on mPRα and γ by analyzing three parameters 1/ their distribution along the mouse nephron and their subcellular location in native kidney, 2/ the ability of progesterone to stimulate ERK pathway and/or Ca^{2+} release from internal stores in native kidney structures and 3/ the cellular localization of mPRα and its molecular determinants in heterologous expression system. We observed that 1/ mPRα expression is restricted to proximal tubules of both male and female mice whereas mPRγ exhibits a much broader expression all along the nephron except the glomerulus, 2/ mPRα and γ are not localized at the plasma membrane in native kidney, 3/ this expression does not permit either progesterone-induced ERK phosphorylation or Ca^{2+} release and 4/ in HEK transfected cells, mPRα localizes in the endoplasmic reticulum (ER) due to a C-terminal ER retention motif (− KXX). Therefore, we have characterized mPRs in kidney but their role in renal physiology remains to be elucidated.

3.1172 Early adhesion induces interaction of FAK and Fyn in lipid domains and activates raft-dependent Akt signaling in SW480 colon cancer cells
Baillat, G., Siret, C., Delamarre, E. and Luis, J.
*Biochim. Biophys. Acta, **1783**, 2323-2331 (2008)*

Integrin-dependent interaction of epithelial tumor cells with extracellular matrix (ECM) is critical for their migration, but also for hematogenous dissemination. Elevated expression and activity of Src family kinases (SFKs) in colon cancer cells is often required in the disease progression. In this work, we highlighted how focal adhesion kinase (FAK) and SFKs interacted and we analyzed how PI3K/Akt and MAPK/Erk1/2 signaling pathways were activated in early stages of colon cancer cell adhesion. During the first hour, integrin engagement triggered FAK-Y397 phosphorylation and a fraction of FAK was located in lipid rafts/caveolae domains where it interacted with Fyn. The FAK-Y861 and/or -Y925 phosphorylations led to a subsequently FAK translocation out of lipid domains. In parallel, a PI3K/Akt pathway dependent of lipid microdomain integrity was activated. In contrast, the MAPK/Erk1/2 signaling triggered by adhesion increased during at least 4 h and was independent of cholesterol disturbing. Thus, FAK/Fyn interaction in lipid microdomains and a Akt-1 activation occurred at the same time during early contact with ECM suggesting a specific signaling dependent of lipid rafts/caveolae domains.

3.1173 Night Blindness and the Mechanism of Constitutive Signaling of Mutant G90D Rhodopsin
Dizhoor, A.M. et al
*J. Neurosci., **28(45)**, 11662-11672 (2008)*

The G90D rhodopsin mutation is known to produce congenital night blindness in humans. This mutation produces a similar condition in mice, because rods of animals heterozygous (D+) or homozygous (D+/+) for this mutation have decreased dark current and sensitivity, reduced Ca^{2+}, and accelerated values of τ_{REC} and τ_D, similar to light-adapted wild-type (WT) rods. Our experiments indicate that G90D pigment activates the cascade, producing an equivalent background light of ~130 Rh* rod^{-1} for D+ and 890 Rh* rod^{-1} for D+/+. The active species of the G90D pigment could be unregenerated G90D opsin or G90D rhodopsin, either spontaneously activated (as Rh*) or in some other form. Addition of 11-*cis*-retinal in lipid vesicles, which produces regeneration of both WT and G90D opsin in intact rods and ROS membranes, had no effect on the waveform or sensitivity of dark-adapted G90D responses, indicating that the active species is not G90D opsin. The noise spectra of dark-adapted G90D and WT rods are similar, and the G90D noise variance is much less than of a WT rod exposed to background light of about the same intensity as the G90D equivalent light, indicating that Rh* is not the active species. We hypothesize that G90D rhodopsin undergoes spontaneous changes in molecular conformation which activate the transduction cascade with low gain. Our experiments provide the first indication that a mutant form of the rhodopsin molecule bound to its 11-*cis*-chromophore can stimulate the visual cascade spontaneously at a rate large enough to produce visual dysfunction.

3.1174 Lamellar Bodies of Human Epidermis: Proteomics Characterization by High Throughput Mass Spectrometry and Possible Involvement of CLIP-170 in their Trafficking/Secretion
Raymonds, A-A-. et al
*Mol. Cell. Proteomics, **7(11)**, 2151-2175 (2008)*

Lamellar bodies (LBs) are tubulovesicular secretory organelles of epithelial cells related to lysosomes. In the epidermis, they play a crucial role in permeability barrier homeostasis, secreting their contents, lipids, a variety of hydrolases, protease inhibitors, and antimicrobial peptides, in the upper keratinocyte layers. The identification of proteins transported in epidermal LBs is still far from complete, and the way their secretion is controlled unknown. In this study, we describe the first proteomics characterization by nano-LC-MS/MS of a fraction enriched in epidermal LBs. We identified 984 proteins, including proteins known or thought to be secreted by LBs. Moreover 31 proteins corresponded to lysosomal components further suggesting that LBs are a new class of secretory lysosomes. Many of the newly found proteins could play a role in the epidermal barrier and desquamation (one acid ceramidase-like protein, apolipoproteins, glycosidases, protease inhibitors, and peptidases) and in LB trafficking (*e.g.* Rab, Arf, and motor complex proteins). We focus here on CLIP-170/restin, a protein that mediates interactions between organelles and microtubules. Western blotting confirmed the presence of CLIP-170 and its known effectors IQGAP1 and Cdc42 in the LB-enriched fraction. We showed, by confocal microscopy analysis of skin cryosections, that CLIP-170 was expressed in differentiated keratinocytes, first at the periphery of the nucleus then with a granular cytoplasmic labeling evocative of LBs. It was preferentially co-localized with Cdc42 and with the known LB protein cathepsin D. CLIP-170 was also largely co-localized with Rab7. This study strongly suggests a new function for CLIP-170, its involvement together with Cdc42 and/or Rab7 in the intracellular trafficking of LBs, and provides evidence that nano-LC-MS/MS combined with monodimensional electrophoresis separation constitutes a powerful method for identifying proteins in a complex mixture such as subcellular structures.

3.1175 **P2Y1 Receptor Activation Elicits Its Partition out of Membrane Rafts and Its Rapid Internalization from Human Blood Vessels: Implications for Receptor Signaling**
Norambuena, A. et al
Mol. Pharmacol., ***74(6)***, *1666-1677 (2008)*

The nucleotide $P2Y_1$ receptor ($P2Y_1R$) is expressed in both the endothelial and vascular smooth muscle cells; however, its plasma membrane microregionalization and internalization in human tissues remain unknown. We report on the role of membrane rafts in $P2Y_1R$ signaling by using sodium carbonate or **OptiPrep** sucrose density gradients, Western blot analysis, reduction of tissue cholesterol content, and vasomotor assays of endothelium-denuded human chorionic arteries. In tissue extracts prepared either in sodium carbonate or **OptiPrep**, approximately 20 to 30% of the total $P2Y_1R$ mass consistently partitioned into raft fractions and correlated with vasomotor activity. Vessel treatment with methyl β-cyclodextrin reduced the raft partitioning of the $P2Y_1R$ and obliterated the $P2Y_1R$-mediated contractions but not the vasomotor responses elicited by either serotonin or KCl. Perfusion of chorionic artery segments with 100 nM 2-methylthio ADP or 10 nM [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-amino-2-(methylthio)-9*H*-purin-9-yl] 2,3dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS 2365), a selective $P2Y_1R$ agonist, not only displaced within 4 min the $P2Y_1R$ localization out of membrane rafts but also induced its subsequent internalization. 2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179), a specific $P2Y_1R$ antagonist, did not cause a similar displacement but blocked the agonist-induced exit from rafts. Neither adenosine nor uridine triphosphate displaced the $P2Y_1R$ from the membrane raft, further evidencing the pharmacodynamics of the receptor-ligand interaction. Vascular reactivity assays showed fading of the ligand-induced vasoconstrictions, a finding that correlated with the $P2Y_1R$ exit from raft domains and internalization. These results demonstrate in intact human vascular smooth muscle the association of the $P2Y_1R$ to membrane rafts, highlighting the role of this microdomain in $P2Y_1R$ signaling.

3.1176 **Hereditary Spastic Paraplegia-Associated Mutations in the NIPA1 Gene and Its Caenorhabditis elegans Homolog Trigger Neural Degeneration In Vitro and In Vivo through a Gain-of-Function Mechanism**
Zhao, J., Matthies, D.S., Botzolakis, E.J., Macdonald, R.L., Blakely, R.D. and Hedera, P.
J. Neurosci., ***28(51)***, *13938-13951 (2008)*

We studied the consequences of expression of wild-type (WT) human NIPA1 and two mutant forms of NIPA1 with known HSP-associated mutations (T45R and G106R) on cultured rat cortical neurons and using equivalent substitutions in the *Caenorhabditis elegans* NIPA1 homolog CeNIPA. WT NIPA1 localized in transfected neuronal and non-neuronal cells to the Golgi complex, a subset of synaptic vesicles, to a subset of early endosomes, and plasma cell membrane. Mutant NIPA1 accumulated in the endoplasmic reticulum (ER) triggering ER stress and features of apoptotic cell death. Flow cytometric analysis of NIPA1 surface expression demonstrated relatively intact trafficking of mutant forms and only the T45R

mutant exhibited modestly reduced patterns of surface expression without evidence for a dominant-negative effect. *In vivo* pan-neuronal expression of the WT *C. elegans* NIPA1 homolog (CeNIPA) was well tolerated, with no obvious impact on neuronal morphology or behavior. In striking contrast, expression of CeNIPA bearing HSP-associated mutations caused a progressive neural degeneration and a clear motor phenotype. Neuronal loss in these animals began at day 7 and by day 9 animals were completely paralyzed. These effects appeared to arise from activation of the apoptotic program triggered by unfolded protein response (UPR), as we observed marked modifications of motor and cellular phenotype when mutant NIPA1 was expressed in caspase (*ced-3*)- and UPR (*xbp-1*)-deficient backgrounds. We propose that HSP-associated mutations in NIPA1 lead to cellular and functional deficits through a gain-of-function mechanism supporting the ER accumulation of toxic NIPA1 proteins.

3.1177 Eosinophil granules function extracellularly as receptor-mediated secretory organelles
Neves, J.S. et al
*PNAS, **105(47)**, 18478-18483 (2008)*

Intracellular granules in several types of leukocytes contain preformed proteins whose secretions contribute to immune and inflammatory functions of leukocytes, including eosinophils, cells notably associated with asthma, allergic inflammation, and helminthic infections. Cytokines and chemokines typically elicit extracellular secretion of granule proteins by engaging receptors expressed externally on the plasma membranes of cells, including eosinophils. Eosinophil granules, in addition to being intracellular organelles, are found as intact membrane-bound structures extracellularly in tissue sites of eosinophil-associated diseases. Neither the secretory capacities of cell-free eosinophil granules nor the presence of functional cytokine and chemokine receptors on membranes of leukocyte granules have been recognized. Here, we show that granules of human eosinophils express membrane receptors for a cytokine, IFN-γ, and G protein–coupled membrane receptors for a chemokine, eotaxin, and that these receptors function by activating signal-transducing pathways within granules to elicit secretion from within granules. Capacities of intracellular granule organelles to function autonomously outside of eosinophils as independent, ligand-responsive, secretion-competent structures constitute a novel postcytolytic mechanism for regulated secretion of eosinophil granule proteins that may contribute to eosinophil-mediated inflammation and immunomodulation.

3.1178 Induction of HIV Transcription by Nef Involves Lck Activation and Protein Kinase Cθ Raft Recruitment Leading to Activation of ERK1/2 but Not NFκB
Witte, V. et al
*J. Immunol., **181**, 8425-8432 (2008)*

The Nef protein of HIV-1 is a key promoter of disease progression, owing to its dramatic yet ill-defined impact on viral replication. Previously, we have shown that Nef enhances embryonic ectodermal development Tat-mediated transcription in a manner depending on Lck and the cytoplasmic sequestration of the transcriptional repressor embryonic ectodermal development. In this study, we report that Lck is activated by Nef and targets protein kinase Cθ downstream, leading to the translocation of the kinase into membrane microdomains. Although microdomain-localized protein kinase Cθ is thought to induce the transcription factor NFκB, we unexpectedly failed to correlate Nef-induced signaling events with enhanced NFκB activity. Instead, we observed an increase in ERK MAPK activity. We conclude that Nef-mediated signaling cooperates with Nef-induced derepression and supports HIV transcription through an ERK MAPK-dependent, but NFκB-independent, pathway.

3.1179 Switch-like Control of SREBP-2 Transport Triggered by Small Changes in ER Cholesterol: A Delicate Balance
Radhakrishnan, A., Goldstein, J.L., McDonald, J.G. and Brown, M.S.
*Cell Metabolism, **8**, 512-521 (2008)*

Animal cells control their membrane lipid composition within narrow limits, but the sensing mechanisms underlying this control are largely unknown. Recent studies disclosed a protein network that controls the level of one lipid—cholesterol. This network resides in the endoplasmic reticulum (ER). A key component is Scap, a tetrameric ER membrane protein that binds cholesterol. Cholesterol binding prevents Scap from transporting SREBPs to the Golgi for activation. Using a new method to purify ER membranes from cultured cells, we show that Scap responds cooperatively to ER cholesterol levels. When ER cholesterol exceeds 5% of total ER lipids (molar basis), SREBP-2 transport is abruptly blocked. Transport resumes

when ER cholesterol falls below the 5% threshold. The 5% threshold is lowered to 3% when cells overexpress Insig-1, a Scap-binding protein. Cooperative interactions between cholesterol, Scap, and Insig create a sensitive switch that controls the cholesterol composition of cell membranes with remarkable precision.

3.1180 Mechanosensing machinery for cells under low substratum rigidity
Wei, W-C., Lin, H-H., Shen, M-R. and Tang, M-J.
*Am. J. Physiol.Cell Physiol., **295**, 1579-1589 (2008)*

Mechanical stimuli are essential during development and tumorigenesis. However, how cells sense their physical environment under low rigidity is still unknown. Here we show that low rigidity of collagen gel downregulates β_1-integrin activation, clustering, and focal adhesion kinase (FAK) Y397 phosphorylation, which is mediated by delayed raft formation. Moreover, overexpression of autoclustered β_1-integrin (V737N), but not constitutively active β_1-integrin (G429N), rescues FAKY397 phosphorylation level suppressed by low substratum rigidity. Using fluorescence resonance energy transfer to assess β_1-integrin clustering, we have found that substratum rigidity between 58 and 386 Pa triggers β_1-integrin clustering in a dose-dependent manner, which is highly dependent on actin filaments but not microtubules. Furthermore, augmentation of β_1-integrin clustering enhances the interaction between β_1-integrin, FAK, and talin. Our results indicate that contact with collagen fibrils is not sufficient for integrin activation. However, substratum rigidity is required for integrin clustering and activation. Together, our findings provide new insight into the mechanosensing machinery and the mode of action for epithelial cells in response to their physical environment under low rigidity.

3.1181 Trafficking of chlamydial antigens to the endoplasmic reticulum of infected epithelial cells
Giles, D.K. and Wyrick, P.B.
*Microbes and Infection, **10**, 1494-1503 (2008)*

Confinement of the obligate intracellular bacterium *Chlamydia trachomatis* to a membrane-bound vacuole, termed an inclusion, within infected epithelial cells neither prevents secretion of chlamydial antigens into the host cytosol nor protects chlamydiae from innate immune detection. However, the details leading to chlamydial antigen presentation are not clear. By immunoelectron microscopy of infected endometrial epithelial cells and in isolated cell secretory compartments, chlamydial major outer membrane protein (MOMP), lipopolysaccharide (LPS) and the inclusion membrane protein A (IncA) were localized to the endoplasmic reticulum (ER) and co-localized with multiple ER markers, but not with markers of the endosomes, lysosomes, Golgi nor mitochondria. Chlamydial LPS was also co-localized with CD1d in the ER. Since the chlamydial antigens, contained in everted inclusion membrane vesicles, were found within the host cell ER, these data raise additional implications for antigen processing by infected uterine epithelial cells for classical and non-classical T cell antigen presentation.

3.1182 The peroxisomal membrane protein import receptor Pex3p is directly transported to peroxisomes by a novel Pex19p- and Pex16p-dependent pathway
Matsuzaki, T. and Fujiki, Y.
*J. Cell Biol., **183(7)**, 1275-1286 (2008)*

Two distinct pathways have recently been proposed for the import of peroxisomal membrane proteins (PMPs): a Pex19p- and Pex3p-dependent class I pathway and a Pex19p- and Pex3p-independent class II pathway. We show here that Pex19p plays an essential role as the chaperone for full-length Pex3p in the cytosol. Pex19p forms a soluble complex with newly synthesized Pex3p in the cytosol and directly translocates it to peroxisomes. Knockdown of Pex19p inhibits peroxisomal targeting of newly synthesized full-length Pex3p and results in failure of the peroxisomal localization of Pex3p. Moreover, we demonstrate that Pex16p functions as the Pex3p-docking site and serves as the peroxisomal membrane receptor that is specific to the Pex3p–Pex19p complexes. Based on these novel findings, we suggest a model for the import of PMPs that provides new insights into the molecular mechanisms underlying the biogenesis of peroxisomes and its regulation involving Pex3p, Pex19p, and Pex16p.

3.1183 Dendrites of Mammalian Neurons Contain Specialized P-Body-Like Structures That Respond to Neuronal Activation
Cougot, N., Bhattacharrya, S.N., Tapia-Arancibia, L., Bordonne, R., Filipowicz, W., Bertrand, E. and Rage, F.
*J. Neurosci., **28(51)**, 13793-13804 (2008)*

Intracellular mRNA transport and local translation play a key role in neuronal physiology. Translationally repressed mRNAs are transported as a part of ribonucleoprotein (RNP) particles to distant dendritic sites, but the properties of different RNP particles and mechanisms of their repression and transport remain largely unknown. Here, we describe a new class of RNP-particles, the dendritic P-body-like structures (dlPbodies), which are present in the soma and dendrites of mammalian neurons and have both similarities and differences to P-bodies of non-neuronal cells. These structures stain positively for a number of P-body and microRNP components, a microRNA-repressed mRNA and some translational repressors. They appear more heterogeneous than P-bodies of HeLa cells, and they rarely contain the exonuclease Xrn1 but are positive for rRNA. These particles show motorized movements along dendrites and relocalize to distant sites in response to synaptic activation. Furthermore, Dcp1a is stably associated with dlP-bodies in unstimulated cells, but exchanges rapidly on neuronal activation, concomitantly with the loss of Ago2 from dlP-bodies. Thus, dlP-bodies may regulate local translation by storing repressed mRNPs in unstimulated cells, and releasing them on synaptic activation.

3.1184 **Proteomics in *Trypanosoma cruzi* – localization of novel proteins to various organelles**
Ferella, M., Nilsson, D., darban, H., Rodrigues, C., Bontempi, E.J., Docampo, R. and Andersson, B.
Proteomics, ***8(13)****, 2735-2749 (2008)*

The completion of the genome sequence of *Trypanosoma cruzi* has been followed by several studies of

protein expression, with the long-term aim to obtain a complete picture of the parasite proteome. We report a proteomic analysis of an organellar cell fraction from *T. cruzi* CL Brener epimastigotes. A total of 396 proteins were identified by LC-MS/MS. Of these, 138 were annotated as hypothetical in the genome databases and the rest could be assigned to several metabolic and biosynthetic pathways, transport, and structural functions. Comparative analysis with a whole cell proteome study resulted in the validation of the expression of 173 additional proteins. Of these, 38 proteins previously reported in other stages were not found in the only large-scale study of the total epimastigote stage proteome. A selected set of identified proteins was analyzed further to investigate gene copy number, sequence variation, transmembrane domains, and targeting signals. The genes were cloned and the proteins expressed with a c-myc epitope tag in *T. cruzi* epimastigotes. Immunofluorescence microscopy revealed the localization of these proteins in different cellular compartments such as ER, acidocalcisome, mitochondrion, and putative cytoplasmic transport or delivery vesicles. The results demonstrate that the use of enriched subcellular fractions allows the detection of *T. cruzi* proteins that are undetected by whole cell proteomic methods.

3.1185 **2-D DIGE analyses of enriched secretory lysosomes reveal heterogeneous profiles of functionally relevant proteins in leukemic and activated human NK cells**
Schmidt, H., Gelhaus, C., Nebendahl, M., Lettau, M., Wartzl, C., Kabelitz, D., Leippe, M. and Janssen, O.
Proteomics, ***8(14)****, 2911-2925 (2008)*

As part of the innate immune system, natural killer (NK) cells detect and lyse tumor and virus-infected cells without prior antigen-dependent recognition and expansion. To this end, they utilize dual-function organelles that combine properties of conventional lysosomes and exocytotic vesicles. Upon stimulation, these secretory lysosomes (SLs) release their cytotoxic molecules into the immunological synapse. In addition, several molecules associated with secretory vesicles become exposed on the plasma membrane. Recent studies often took advantage of the few established NK cell lines, for instance to analyze the exocytotic machinery associated with NK cell vesicles. NK cell lines and primary NK cells differ, however, substantially in the expression of "typical" surface receptors and their requirements to induce target cell lysis. Here, we directly compared the lysosomal compartments of different NK cell populations. We enriched SLs of two leukemic cell lines (YTS and NKL) and IL-2-expanded NK cells by subcellular fractionation and characterized their proteome by 2-D difference gel electrophoresis and MS. Although the overall protein composition of the lysosomal preparations was very similar and more than 90% of the proteins were present at comparable levels, we define a cell line-specific setup of functionally relevant proteins involved in antigen presentation and cytotoxic effector function.

3.1186 **Sub-cellular localization of membrane proteins**
Sadowski, P.G., Groen, A.J., Dupree, P. and Lilley, K.S.
Proteomics, ***8(19)****, 3991-4011 (2008)*

In eukaryotes, numerous complex sub-cellular structures exist. The majority of these are delineated by membranes. Many proteins are trafficked to these in order to be able to carry out their correct physiological function. Assigning the sub-cellular location of a protein is of paramount importance to biologists in the elucidation of its role and in the refinement of knowledge of cellular processes by tracing certain activities to specific organelles. Membrane proteins are a key set of proteins as these form part of the boundary of the organelles and represent many important functions such as transporters, receptors, and trafficking. They are, however, some of the most challenging proteins to work with due to poor solubility, a wide concentration range within the cell and inaccessibility to many of the tools employed in proteomics studies. This review focuses on membrane proteins with particular emphasis on sub-cellular localization in terms of methodologies that can be used to determine the accurate location of membrane proteins to organelles. We also discuss what is known about the membrane protein cohorts of major organelles.

3.1187 **Src-Tyrosine kinases are major agents in mitochondrial tyrosine phosphorylation**
Tibaldi, E., Brunati, A.M., Massimino, M.L., Stringaro, A., Colone, M., Agostinelli, e., Arancia, G. and Toninello, A.
J. Cell. Biochem., ***104(3)***, *840-849 (2008)*

Mitochondrial tyrosine phosphorylation is emerging as an important mechanism in regulating mitochondrial function. This article, aimed at identifying which kinases are the major agents in mitochondrial tyrosine phosphorylation, shows that this role should be attributed to Src family members. Indeed, various members of this family, for example, Fgr, Fyn, Lyn, c-Src, are constitutively present in the internal structure of mitochondria as well as Csk, a key enzyme in the regulation of the activity of this family. By means of different approaches, biochemical fractioning, Western blotting and immunogold analysis “in situ” of phosphotyrosine signaling, evidence is reported on the existence of a signal transduction pathway from plasma membrane to mitochondria, resulting in increasing Src-dependent mitochondrial tyrosine phosphorylation. The activation of Src kinases at mitochondrial level is associated with the proliferative status where several mitochondrial proteins are specifically tyrosine-phosphorylated.

3.1188 **Low substratum rigidity of collagen gel promotes ERK phosphorylation via lipid raft to augment cell migration**
Wei, W-C., Hsu, Y-C., Chiu, W-T., Wang, C-Z., Wu, C-M., Wang, Y-K., Shen, M-R. and Tang, M-J.
J. Cell. Biochem., ***103(4)***, *1111-1124 (2008)*

Previous study demonstrated that low substratum rigidity down-regulates focal adhesion proteins. In this study we found that cells cultured on collagen gel exhibited higher migration capacity than those cultured on collagen gel-coated dishes. Low rigidity of collagen gel induced delayed but persistent phosphorylation of ERK1/2. Inhibition of collagen gel-induced ERK1/2 phosphorylation by MEK inhibitors and ERK2 kinase mutant induced a rounding up of the cells and prevented collagen gel-induced cell migration. Interestingly, phosphorylated ERK1/2 induced by low rigidity was present in focal adhesion sites and the lipid raft. MβCD (Methyl-β-cyclodextrin), a lipid raft inhibitor, inhibited collagen gel-induced ERK1/2 phosphorylation, and cell migration. Overexpression of FAK C-terminal fragment (FRNK) in MDCK cells triggered ERK phosphorylation. Meanwhile, low substratum rigidity induced degradation of FAK into a 35 kDa C-terminal fragment. A calpain inhibitor that partially rescued FAK degradation also prevented low rigidity-induced ERK phosphorylation. However, MβCD did not prevent low rigidity-induced FAK degradation. Taken together, we demonstrate that the degradation product of FAK induced by collagen gel triggers activation of ERK1/2, which in turn facilitates cell spreading and migration through the lipid raft.

3.1189 *Escherichia coli* **interaction with human brain microvascular endothelial cells induces signal transducer and activator of transcription 3 association with the C-terminal domain of Ec-gp96, the outer membrane protein A receptor for invasion**
Maruvada, R., Argon, Y. and Prasadaroa, N.
Cell. Microbiol., ***10(11)***, *2326-2338 (2008)*

Our inability to develop new therapeutic strategies to prevent meningitis due to *Escherichia coli* K1 is attributed to our incomplete understanding of the pathophysiology of the disease. Previously, we demonstrated that outer membrane protein A of *E. coli* interacts with a gp96 homologue, Ec-gp96, on human brain microvascular endothelial cells (HBMEC) for invasion. However, signalling events mediated by Ec-gp96 that allow internalization of *E. coli* are incompletely understood. Here, we demonstrate that signal transducer and activator of transcription 3 (Stat3) activation and its interaction with Ec-gp96 were critical for *E. coli* invasion. The activated Stat3 was colocalized with Ec-gp96 at the actin condensation

sites, and overexpressing a dominant negative (DN) form of Stat3 in HBMEC significantly abrogated the invasion. Furthermore, overexpression of Ec-gp96Δ200, the C-terminal 214-amino-acid truncated Ec-gp96, prevented the invasion of *E. coli* in HBMEC. In contrast, lack of ATP binding by gp96 did not affect the invasion. Overexpression of DN forms of either phosphatidyl inositol-3 kinase (PI3-kinase) subunit p85 or protein kinase C-α (PKC-α) had no effect on the activation of Stat3 and its association with Ec-gp96, whereas overexpression of DN-Stat3 abolished the activation of both PI3-kinase and PKC-α. Together, our findings identified a novel interaction of Stat3 with Ec-gp96, upstream of PI3-kinase and PKC-α activation that is required for the invasion of *E. coli* into HBMEC.

3.1190 Functional interactions between anthrax toxin receptors and the WNT signalling protein LRP6
Abrami, L., Kunz, B., Deuquet, J., Bafico, A., Davidson, G and Giscoe van der Goot, F.
*Cell. Microbiol., **10(12)**, 2509-2519 (2008)*

To exert its activity, anthrax toxin must be endocytosed and its enzymatic toxic subunits delivered to the cytoplasm. It has been proposed that, in addition to the anthrax toxin receptors (ATRs), lipoprotein-receptor-related protein 6 (LRP6), known for its role in Wnt signalling, is also required for toxin endocytosis. These findings have however been challenged. We show that LRP6 can indeed form a complex with ATRs, and that this interaction plays a role both in Wnt signalling and in anthrax toxin endocytosis. We found that ATRs control the levels of LRP6 in cells, and thus the Wnt signalling capacity. RNAi against ATRs indeed led to a drastic decrease in LRP6 levels and a subsequent drop in Wnt signalling. Conversely, LRP6 plays a role in anthrax toxin endocytosis, but is not essential. We indeed found that toxin binding triggered tyrosine phosphorylation of LRP6, induced its redistribution into detergent-resistant domains, and its subsequent endocytosis. RNAis against LRP6 strongly delayed toxin endocytosis. As the physiological role of ATRs is probably to interact with the extracellular matrix, our findings raise the interesting possibility that, through the ATR–LRP6 interaction, adhesion to the extracellular matrix could locally control Wnt signalling.

3.1191 Caveolin-1 secreting LNCaP cells induce tumor growth of caveolin-1 negative LNCaP cells *in vivo*
Bartz, R., Zhou, J., Hsieh, J-T., Ying, Y., Li, W. and Liu, P.
*Int. J. Cancer, **122(3)**, 520-525 (2008)*

Caveolin-1 (Cav-1) was originally identified as a structural protein of caveolae, which is a plasma membrane domain that regulates a variety of signaling pathways involved in cell growth and migration. Here, we show that expression of Cav-1 in the Cav-1-deficient human prostate cancer cell line LNCaP both stimulates cell proliferation and promotes tumor growth in nude mice. Unexpectedly, Cav-1 expressing LNCaP ($LNCaP^{Cav\text{-}1}$) cells injected into one side of a nude mouse promoted tumor growth of Cav-1 negative LNCaP cells injected on the contralateral side of the same animal. The LNCaP tumors were positive for Cav-1, however, this signal was not caused by migrated $LNCaP^{Cav\text{-}1}$ cells, but we show that this Cav-1 was secreted by the $LNCaP^{Cav\text{-}1}$ tumors. We demonstrate that conditioned media from $LNCaP^{Cav\text{-}1}$ cells contained Cav-1 that was associated with a lipoprotein particle ranging in size from 15 to 30 nm and a density similar to high density lipoprotein particle. These results suggest that $LNCaP^{Cav\text{-}1}$ cells secreting Cav-1 particle produce an endocrine factor that stimulates tumor growth.

3.1192 A flow-cytometry method for analyzing the composition of membrane rafts
Morales-Garzia, M.G., Fournie, J-J., Morena-Altamirano, M.M.B., Rodriguez-Luna, G., Mondragon-Flores, R. and Sanchez-Garzia, F.J.
*Cytometry Part A, **73A(10),** 918-925 (2008)*

Membrane rafts are involved in a broad variety of biological processes. Their protein composition under growth factor stimulation, anti-inflammatory or proinflammatory microenvironments, or in the course of pathogenic infections still remains to be determined. However, current techniques aimed at the identification of particular proteins on membrane rafts are not devoid of pitfalls. Membrane rafts were obtained by detergent-free based differential centrifugation from Jurkat T cells and J774 macrophages. Membrane rafts were labeled with fluorochrome-labeled antibodies directed against different cell membrane molecules, and with fluorochrome-labeled cholera toxin B that targets GM1 and analyzed by flow cytometry. CD3, CD11a, and GM1 were shown to be differentially expressed on Jurkat T cell-derived membrane rafts, indicating heterogeneity in membrane rafts composition. On the other hand, it was shown in J774 cell-derived membrane rafts that most but not all CD14 is present in the GM1-containing membrane fragments, thus confirming the heterogeneity of membrane rafts composition in other cell lines. The method described here allows the fluorometric assessment of the relative expression of more than one

membrane raft component at a time, and at a single vesicle level in a fast and sensitive manner. This method seems to be a suitable approach to evaluate the molecular heterogeneity in membrane rafts composition.

3.1193 Proteomic and immunologic analyses of brain tumor exosomes
Graner, M.W., Alzate, O., Dechkovskaia, A.M., Keene, J.D., sampson, J.H., Mitchell, D.A. and Bigner, D.D.
FASEB J., ***23(5)****, 1541-1557 (2009)*

Brain tumors are horrific diseases with almost universally fatal outcomes; new therapeutics are desperately needed and will come from improved understandings of glioma biology. Exosomes are endosomally derived 30–100 nm membranous vesicles released from many cell types into the extracellular milieu; surprisingly, exosomes are virtually unstudied in neuro-oncology. These microvesicles were used as vaccines in other tumor settings, but their immunological significance is unevaluated in brain tumors. Our purpose here is to report the initial biochemical, proteomic, and immunological studies on murine brain tumor exosomes, following known procedures to isolate exosomes. Our findings show that these vesicles have biophysical characteristics and proteomic profiles similar to exosomes from other cell types but that brain tumor exosomes have unique features (*e.g.*, very basic isoelectric points, expressing the mutated tumor antigen EGFRvIII and the putatively immunosuppressive cytokine TGF-β). Administration of such exosomes into syngeneic animals produced both humoral and cellular immune responses in immunized hosts capable of rejecting subsequent tumor challenges but failed to prolong survival in established orthotopic models. Control animals received saline or cell lysate vaccines and showed no antitumor responses. Exosomes and microvesicles isolated from sera of patients with brain tumors also possess EGFR, EGFRvIII, and TGF-β. We conclude that exosomes released from brain tumor cells are biochemically/biophysically like other exosomes and have immune-modulating properties. They can escape the blood-brain barrier, with potential systemic and distal signaling and immune consequences

3.1194 Use of Bodipy-labeled sphingolipid and cholesterol analogs to examine membrane microdomains in cells
Marks, D.L., Bittman, r. and pagano, R.E.
Histochem. Cell. Biol., ***130(5)****, 819-832 (2008)*

Much evidence has accumulated to show that cellular membranes such as the plasma membrane, contain multiple “microdomains” of differing lipid and protein composition and function. These domains are sometimes enriched in cholesterol and sphingolipids and are believed to be important structures for the regulation of many biological and pathological processes. This review focuses on the use of fluorescent (Bodipy) labeled analogs of sphingolipids and cholesterol to study such domains. We discuss the similarities between the behavior of Bodipy-cholesterol and natural cholesterol in artificial bilayers and in cultured cells, and the use of Bodipy-sphingolipid analogs to visualize membrane domains in living cells based on the concentration-dependent monomer-excimer fluorescence properties of the Bodipy-fluorophore. The use of Bodipy-d-erythro-lactosylceramide is highlighted for detection of domains on the plasma membrane and endosome membranes, and the importance of the sphingolipid stereochemistry in modulating domain formation is discussed. Finally, we suggest that Bodipy-sphingolipids may be useful in future studies to examine the relationship between membrane domains at the cell surface and domains enriched in other lipids and proteins on the inner leaflet of the plasma membrane.

3.1195 A deleted prion protein that is neurotoxic in vivo is localized normally in cultured cells
Christensen, H.M. and Harris, D.A.
J. Neurochem., ***108****, 44-56 (2009)*

The prion protein (PrP) possesses sequence-specific domains that endow the molecule with neuroprotective and neurotoxic activities, and that may contribute to the pathogenesis of prion diseases. To further define critical neurotoxic determinants within PrP, we previously generated Tg([DELTA]CR) mice that express a form of PrP harboring a deletion of 21 amino acids within the central domain of the protein [Li et al., EMBO J. 26 (2007), 548]. These animals exhibit a neonatal lethal phenotype that is dose-dependently rescued by co-expression of wild-type PrP. In this study, we examined the localization and cell biological properties of the PrP([DELTA]CR) protein in cultured cells to further understand the mechanism of PrP([DELTA]CR) neurotoxicity. We found that the distribution of PrP([DELTA]CR) was identical to that of wild-type PrP in multiple cell lines of both neuronal and non-neuronal origin, and that co-expression of the two proteins did not alter the localization of either one. Both proteins were found in

lipid rafts, and both were localized to the apical surface in polarized epithelial cells. Taken together, our results suggest that PrP([DELTA]CR) toxicity is not a result of mislocalization or aggregation of the protein, and more likely stems from altered binding interactions leading to the activation of deleterious signaling pathways.

3.1196 De novo generation of a transmissible spongiform encephalopathy by mouse transgenesis
Sigurdson, C.J. et al
*PNAS, **106(1)**, 304-309 (2009)*

Most transmissible spongiform encephalopathies arise either spontaneously or by infection. Mutations of *PRNP*, which encodes the prion protein, PrP, segregate with phenotypically similar diseases. Here we report that moderate overexpression in transgenic mice of mPrP(170N,174T), a mouse PrP with two point mutations that subtly affect the structure of its globular domain, causes a fully penetrant lethal spongiform encephalopathy with cerebral PrP plaques. This genetic disease was reproduced with 100% attack rate by intracerebral inoculation of brain homogenate to *tga20* mice overexpressing WT PrP, and from the latter to WT mice, but not to PrP-deficient mice. Upon successive transmissions, the incubation periods decreased and PrP became more protease-resistant, indicating the presence of a strain barrier that was gradually overcome by repeated passaging. This shows that expression of a subtly altered prion protein, with known 3D structure, efficiently generates a prion disease.

3.1197 Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially
Spencer, L.A., Szelaq, C.T., Perez, S.A.C., Kirschhofer, C.L., Neves, J.S., Radke, A.L and Weller, P.F.
*J. Leukoc. Biol., **85**, 117-123 (2009)*

Eosinophils are innate immune leukocytes implicated in the initiation and maintenance of type 2 immune responses, including asthma and allergy. The ability to store and rapidly secrete preformed cytokines distinguishes eosinophils from most lymphocytes, which must synthesize cytokine proteins prior to secretion and may be a factor in the apparent Th2 bias of eosinophils. Multiple studies confirm that human eosinophils from atopic or hypereosinophilic donors can secrete over 30 cytokines with a varying and often opposing immune-polarizing potential. However, it remains unclear whether all of these cytokines are constitutively preformed and available for rapid secretion from eosinophils in the circulation of healthy individuals or are restricted to eosinophils from atopic donors. Likewise, the relative concentrations of cytokines stored within eosinophils have not been studied. Here, we demonstrate that human blood eosinophils are not singularly outfitted with Th2-associated cytokines but rather, constitutively store a cache of cytokines with nominal Th1, Th2, and regulatory capacities, including IL-4, IL-13, IL-6, IL-10, IL-12, IFN-γ, and TNF-α. We demonstrate further rapid and differential release of each cytokine in response to specific stimuli. As agonists, strong Th1 and inflammatory cytokines elicited release of Th2-promoting IL-4 but not Th1-inducing IL-12. Moreover, a large quantity of IFN-γ was secreted in response to Th1, Th2, and inflammatory stimuli. Delineations of the multifarious nature of preformed eosinophil cytokines and the varied stimulus-dependent profiles of rapid cytokine secretion provide insights into the functions of human eosinophils in mediating inflammation and initiation of specific immunity.

3.1198 An Amphipathic α-Helix Controls Multiple Roles of Brome Mosaic Virus Protein 1a in RNA Replication Complex Assembly and Function
Liu, L., Westler, W.M., den Boon, J.A., Wang, X., Diaz, A., Steinberg, H.A. and Ahlquist, P.
*PloSPathogens, **5(3)**, e1000351 (2009)*

Brome mosaic virus (BMV) protein 1a has multiple key roles in viral RNA replication. 1a localizes to perinuclear endoplasmic reticulum (ER) membranes as a peripheral membrane protein, induces ER membrane invaginations in which RNA replication complexes form, and recruits and stabilizes BMV 2a polymerase ($2a^{Pol}$) and RNA replication templates at these sites to establish active replication complexes. During replication, 1a provides RNA capping, NTPase and possibly RNA helicase functions. Here we identify in BMV 1a an amphipathic α-helix, helix A, and use NMR analysis to define its structure and propensity to insert in hydrophobic membrane-mimicking micelles. We show that helix A is essential for efficient 1a–ER membrane association and normal perinuclear ER localization, and that deletion or mutation of helix A abolishes RNA replication. Strikingly, mutations in helix A give rise to two dramatically opposite 1a function phenotypes, implying that helix A acts as a molecular switch regulating the intricate balance between separable 1a functions. One class of helix A deletions and amino acid substitutions markedly inhibits 1a–membrane association and abolishes ER membrane invagination, viral

RNA template recruitment, and replication, but doubles the 1a-mediated increase in $2a^{Pol}$ accumulation. The second class of helix A mutations not only maintains efficient 1a–membrane association but also amplifies the number of 1a-induced membrane invaginations 5- to 8-fold and enhances viral RNA template recruitment, while failing to stimulate $2a^{Pol}$ accumulation. The results provide new insights into the pathways of RNA replication complex assembly and show that helix A is critical for assembly and function of the viral RNA replication complex, including its central role in targeting replication components and controlling modes of 1a action.

3.1199 Analysis of the Differential Host Cell Nuclear Proteome Induced by Attenuated and Virulent Hemorrhagic Arenavirus Infection
Bowick, G.C., Spratt, H.M., Hogg, A.E., Endsley, J.J., Wiktorowicz, J.E., Kurosky, A., Luxon, B.A., Gorenstein, D.G. and Herzog, N.K.
J. Virol., ***83(2)***, *687-700 (2009)*

Arenaviruses are important emerging pathogens and include a number of hemorrhagic fever viruses classified as NIAID category A priority pathogens and CDC potential biothreat agents. Infection of guinea pigs with the New World arenavirus *Pichindé virus* (PICV) has been used as a biosafety level 2 model for the *Lassa virus*. Despite continuing research, little is known about the molecular basis of pathogenesis, and this has hindered the design of novel antiviral therapeutics. Modulation of the host response is a potential strategy for the treatment of infectious diseases. We have previously investigated the global host response to attenuated and lethal arenavirus infections by using high-throughput immunoblotting and kinomics approaches. In this report, we describe the differential nuclear proteomes of a murine cell line induced by mock infection and infection with attenuated and lethal variants of PICV, investigated by using two-dimensional gel electrophoresis. Spot identification using tandem mass spectrometry revealed the involvement of a number of proteins that regulate inflammation via potential modulation of NF-κB activity and of several heterogeneous nuclear ribonuclear proteins. Pathway analysis revealed a potential role for transcription factor XBP-1, a transcription factor involved in major histocompatibility complex II (MHC-II) expression; differential DNA-binding activity was revealed by electrophoretic mobility shift assay, and differences in surface MHC-II expression were seen following PICV infection. These data are consistent with the results of several previous studies and highlight potential differences between transcriptional and translational regulation. This study provides a number of differentially expressed targets for further research and suggests that key events in pathogenesis may be established early in infection.

3.1200 Intracellular Signaling Mechanisms and Activities of Human Herpesvirus 8 Interleukin-6
Chen, D., Sandford, G. and Nicholas, J.
J. Virol., ***83(2)***, *722-733 (2009)*

Human herpesvirus 8 (HHV-8)-encoded viral interleukin-6 (vIL-6) has been implicated as a key factor in virus-associated neoplasia because of its proproliferative and survival effects and also in view of its angiogenic properties. A major difference between vIL-6 and human IL-6 (hIL-6) is that vIL-6, uniquely, is largely retained and can signal intracellularly. While vIL-6 is generally considered to be a lytic gene, several reports have noted its low-level expression in latently infected primary effusion lymphoma (PEL) cultures, in the absence of other lytic gene expression. Thus, intracellular autocrine signal transduction by the viral cytokine may be of particular relevance to the growth and survival of latently infected cells and to pathogenesis. Here we report that most intracellular vIL-6 is located in the endoplasmic reticulum (ER), signals via the gp130 signal transducer in this compartment, and does so independently of the gp80 α-subunit of the IL-6 receptor, required for hIL-6 signal transduction. Signaling and biological assays incorporating ER-retained vIL-6 and hIL-6 confirmed vIL-6 activity, specifically, in this compartment. Knockdown of vIL-6 expression in PEL cells led to markedly reduced cell growth in normal culture, independently of extracellular cytokines. This could be reversed by reintroduction via virus vector of exclusively ER-retained vIL-6. These data indicate that in virus biology vIL-6 may act to support the growth and survival of cells latently infected with HHV-8 in an autocrine manner via intracrine signaling and that these activities may contribute to the maintenance of latently infected cells and to virus-induced neoplasia.

3.1201 Novel N-terminal Cleavage of APP Precludes Aβ Generation in ACAT-Defective AC29 Cells
Huttunen, H.J., Puglielli, L., Ellis, B.C., MacKenzie Ingano, L.A. and Kovacs, D.M.
J. Mol. Neurosci., ***37(6)***, *6-15 (2009)*

A common pathogenic event that occurs in all forms of Alzheimer's disease is the progressive accumulation of amyloid β-peptide (Aβ) in brain regions responsible for higher cognitive functions. Inhibition of acyl-coenzyme A: cholesterol acyltransferase (ACAT), which generates intracellular cholesteryl esters from free cholesterol and fatty acids, reduces the biogenesis of the Aβ from the amyloid precursor protein (APP). Here we have used AC29 cells, defective in ACAT activity, to show that ACAT activity steers APP either toward or away from a novel proteolytic pathway that replaces both α and the amyloidogenic β cleavages of APP. This alternative pathway involves a novel cleavage of APP holoprotein at Glu281, which correlates with reduced ACAT activity and Aβ generation in AC29 cells. This sterol-dependent cleavage of APP occurs in the endosomal compartment after internalization of cell surface APP. The resulting novel C-terminal fragment APP-C470 is destined to proteasomal degradation limiting the availability of APP for the Aβ generating system. The proportion of APP molecules that are directed to the novel cleavage pathway is regulated by the ratio of free cholesterol and cholesteryl esters in cells. These results suggest that subcellular cholesterol distribution may be an important regulator of the cellular fate of APP holoprotein and that there may exist several competing proteolytic systems responsible for APP processing within the endosomal compartment.

3.1202 Analysis of Viral and Cellular Proteins in HIV-1 Reverse Transcription Complexes by Co-immunoprecipitation
Iordanskiy, S.N. and Bukrinsky, M.I.
Methods in Mol. Biol., ***485****, 121-134 (2009)*

Molecular details and temporal organization of the early (preintegration) phase of HIV life cycle remain among the least investigated and most controversial problems in the biology of HIV. To accomplish reverse transcription and intracellular transport of the viral genetic material, HIV forms multi-molecular complexes termed reverse transcription complexes (RTCs). Analysis of the kinetics of reverse transcription and nuclear import of RTCs, as well as assessment of the changes in their protein content in the course of reverse transcription and nuclear translocation is a necessary step in understanding the mechanisms of cytoplasmic maturation and nuclear import of HIV-1 RTCs. Here, we review methods that allow quantitative assessment of the dynamics of the maturation of HIV-1 RTCs and transformations of RTC protein composition associated with nuclear import of the complexes.

3.1203 Proteomics Study of the Hepatitis C Virus Replication Complex
Chang, K., Wang, T. and Luo, G.
Methods in Mol. Biol., ***510****, 185-193 (2009)*

RNA replication of HCV occurs in the multiprotein complexes associated with the endoplasmic reticular (ER) membranes. The HCV NS3 to NS5B proteins are necessary and sufficient for HCV RNA replication in the cell, but cellular proteins in the HCV replication complex (RC) have not been determined. Several methods have been used to isolate the HCV RC, including crude cell extract preparation, subcellular fractionation, and affinity purification. The components of the HCV RC can be separated by two-dimensional electrophoresis and then determined by proteolytical digestion and mass spectrometry analysis in conjunction with peptide/protein database search and immunobiochemistry and functional genomic studies.

3.1204 Proteins Associated with Immunopurified Granules from a Model Pancreatic Islet β-Cell System: Proteomic Snapshot of an Endocrine Secretory Granule
Hickey, A.J.R., Bradley, J.W.I., Skea, G.L., Middleditch, M.J., Buchanan, C.M., Phillips, A.R.J. and Cooper, G.J.S.
J. Proteome Res., ***8(1)****, 178-186 (2009)*

β-Cell granules contain proteins involved in fuel regulation, which when altered, contribute to metabolic disorders including diabetes mellitus. We analyzed proteins present in purified granules from the INS-1E β-cell model. Fifty-one component proteins were identified by LC-MS/MS including hormones, granins, protein processing components, cellular trafficking components, enzymes implicated in cellular metabolism and chaperone proteins. These findings may increase understanding of granule secretion and the processes leading to protein aggregation and β-cell death in type-2 diabetes.

3.1205 Ca^{2+} influx mechanisms in caveolae vesicles of pulmonary smooth muscle plasma membrane under inhibition of $\alpha_2\beta_1$ isozyme of Na^+/K^+-ATPase by ouabain
Ghosh, B., Kar, P., Mandal, A., Dey, K., Chakraborti, T. And Chakraborti, S.

*Life Sciences, **84**, 139-148 (2009)*

Aims
We sought to determine the mechanisms of an increase in Ca^{2+} level in caveolae vesicles in pulmonary smooth muscle plasma membrane during Na^+/K^+-ATPase inhibition by ouabain.
Main methods
The caveolae vesicles isolated by density gradient centrifugation were characterized by electron microscopic and immunologic studies and determined ouabain induced increase in Na^+ and Ca^{2+} levels in the vesicles with fluorescent probes, SBFI-AM and Fura2-AM, respectively.
Key findings
We identified the $\alpha_2\beta_1$ and $\alpha_1\beta_1$ isozymes of Na^+/K^+-ATPase in caveolae vesicles, and only the $\alpha_1\beta_1$ isozyme in noncaveolae fraction of the plasma membrane. The α_2-isoform contributes solely to the enzyme inhibition in the caveolae vesicles at 40 nM ouabain. Methylisobutylamiloride (Na^+/H^+-exchange inhibitor) and tetrodotoxin (voltage-gated Na^+-channel inhibitor) pretreatment prevented ouabain induced increase in Na^+ and Ca^{2+} levels. Ouabain induced increase in Ca^{2+} level was markedly, but not completely, inhibited by KB-R7943 (reverse-mode Na^+/Ca^{2+}-exchange inhibitor) and verapamil (L-type Ca^{2+}-channel inhibitor). However, pretreatment with tetrodotoxin in conjunction with KB-R7943 and verapamil blunted ouabain induced increase in Ca^{2+} level in the caveolae vesicles, indicating that apart from Na^+/Ca^+-exchanger and L-type Ca^{2+}-channels, "slip-mode conductance" of Na^+ channels could also be involved in this scenario.
Significance
Inhibition of α_2 isoform of Na^+/K^+-ATPase by ouabain plays a crucial role in modulating the Ca^{2+} influx regulatory components in the caveolae microdomain for marked increase in $(Ca^{2+})_i$ in the smooth muscle, which could be important for the manifestation of pulmonary hypertension.

3.1206 Moesin Regulates the Trafficking of Nascent Clathrin-coated Vesicles
Barroso-Gonzales, J., Machado, J-D., Garcia-Exposito, L. and Valenzuela-Fernandez, A.
*J. Biol. Chem., **284(4)**, 2419-2434 (2009)*

Clathrin-coated vesicles are responsible for the trafficking of several internalized biological cargos. We have observed that the endogenous F-actin-linker moesin co-distributes with constitutive components of clathrin-coated structures. Total internal reflection fluorescence microscopy studies have shown that short interference RNA of moesin enhances the lateral movement of clathrin-coated structures and provokes their abnormal clustering. The aggregation of clathrin-coated structures has also been observed in cells overexpressing N-moesin, a dominant-negative construct unable to bind to F-actin. Only overexpressed moesin constructs with an intact phosphatidylinositol 4,5-bisphosphate-binding domain co-distribute with clathrin-coated structures. Hence, this N-terminal domain is mostly responsible for moesin/clathrin-coated structure association. Biochemical endosome fractioning together with total internal reflection fluorescence microscopy comparative studies, between intact cells and plasma-membrane sheets, indicate that moesin knockdown provokes the accumulation of endocytic rab5-clathrin-coated vesicles carrying the transferrin receptor. The altered trafficking of these endocytic rab5-clathrin-coated vesicles accounts for a transferrin receptor recycling defect that reduces cell-surface expression of the transferrin receptor and increases the amount of sequestered transferrin ligand. Therefore, we propose that moesin is a clathrin-coated vesicle linker that drives cargo trafficking and acts on nascent rab5-clathrin-coated vesicles by simultaneously binding to clathrin-coated vesicle-associated phosphatidylinositol 4,5-bisphosphate and actin cytoskeleton. Hence, functional alterations of moesin may be involved in pathological disorders associated with clathrin-mediated internalization or receptor recycling.

3.1207 Kinesin Adapter JLP Links PIKfyve to Microtubule-based Endosome-to-Trans-Golgi Network Traffic of Furin
Ikonomov, O.C., Fligger, J., Sbrissa, D., Dondapati, R., Mlak, K., Deebn, R. And Shisheva, A.
*J. Biol. Chem., **284(6)**, 3750-3761 (2009)*

JIPs (c-Jun N-terminal kinase interacting proteins), which scaffold JNK/p38 MAP kinase signaling modules, also bind conventional kinesins and are implicated in microtubule-based membrane trafficking in neuronal cells. Here we have identified a novel splice variant of the *Jip4* gene product JLP_L (JNK-interacting leucine zipper protein) in yeast-two hybrid screens with the phosphoinositide kinase PIKfyve. The interaction was confirmed by pulldown and coimmunoprecipitation assays in native cells. It engages the PIKfyve cpn60_TCP1 consensus sequence and the last 75 residues of the JLP C terminus. Subpopulations of both proteins cofractionated and populated similar structures at the cell perinuclear region. Because PIKfyve is essential in endosome-to-trans-Golgi network (TGN) cargo transport, we tested

whether JLP is a PIKfyve functional partner in this trafficking pathway. Short interfering RNA (siRNA)-mediated depletion of endogenous JLP or PIKfyve profoundly delayed the microtubule-based transport of chimeric furin (Tac-furin) from endosomes to the TGN in a CHO cell line, which was rescued upon ectopic expression of siRNA-resistant JLP or PIKfyve constructs. Peptides from the contact sites in PIKfyve and JLP, or a dominant-negative PIKfyve mutant introduced into cells by ectopic expression or microinjection, induced a similar defect. Because Tac-TGN38 delivery from endosomes to the TGN, unlike that of Tac-furin, does not require intact microtubules, we monitored the effect of JLP and PIKfyve depletion or the interacting peptides administration on Tac-TGN38 trafficking. Remarkably, neither maneuver altered the Tac-TGN38 delivery to the TGN. Our data indicate that JLP interacts with PIKfyve and that both proteins and their association are required in microtubule-based, but not in microtubule-independent, endosome-to-TGN cargo transport.

3.1208 Stabilizing effects of eicosapentaenoic acid on Kv1.5 channel protein expressed in mammalian cells
Koshida, S. et al
*Eur. J. Pharmacol., **604**, 93-102 (2009)*

We investigated the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the stability of Kv1.5 channel protein. The expression and function of Kv1.5 (Kv1.5-FLAG) in transfected African green monkey kidney fibroblast cells as well as rat atrium were estimated by immunoblotting, immunoprecipitation, immunofluorescence and patch-clamp techniques. Both EPA and DHA immediately blocked Kv1.5 channel current in a dose-dependent manner, accompanied by reduction of their phosphorylation. Chronic treatment (for 12 h) with EPA at lower concentrations (0.3–10 μM) increased the level of Kv1.5-FLAG protein as well as Kv1.5 channel current without changes in its gating kinetics, prolonging its half-life; in contrast, both EPA and DHA at higher concentrations (30–100 μM) decreased the expression of Kv1.5-FLAG. EPA at the higher concentrations also decreased mRNA of Kv1.5 and synapse-associated protein 97 expression. EPA at the lower concentrations increased Kv1.5 expression in the endoplasmic reticulum, Golgi apparatus and cell membrane. EPA-induced increase of Kv1.5 channel expression and current was abolished by pretreatment with the protein transport inhibitor brefeldin A or colchicines, and by the Kv1.5 channel blocker 4-aminopyridine. Oral administration of EPA (30 mg/kg) increased the level of endogenous Kv1.5 in rat atria. These results indicate that chronic treatment with EPA at lower concentrations stabilizes Kv1.5 channel protein in the endoplasmic reticulum and Golgi apparatus thereby enhancing the Kv1.5 channel current on the cell membrane.

3.1209 The N-terminal half of the receptor domain of botulinum neurotoxin A binds to microdomains of the plasma membrane
Muraro, L., Tosatto, S., Motterlini, L., Rosetto, O. and Montecucco, C.
*Biochem. Biophys. Res. Comm., **380**, 76-80 (2009)*

Botulinum neurotoxin type A (BoNT/A) is largely employed in human therapy because of its specific inhibition of peripheral cholinergic nerve terminals. BoNT/A binds to them rapidly and with high specificity via its receptor binding domain termed HC. Recent evidence indicate that BoNT/A interacts specifically with polysialogangliosides and with a luminal loop of the synaptic vesicle protein SV2 via the C-terminal half of HC.
Here we show that the N-terminal half of HC binds to sphingomyelin-enriched membrane microdomains and that it has a defined interaction with phosphatidylinositol phosphates (PIP). We have identified a PIP binding site in this half of HC and we show how this interaction could predispose BoNT/A for membrane insertion, which is the step subsequent to binding, in the four-steps route leading BoNT/A inside nerve terminals.

3.1210 Biochemical and proteomic approaches for the study of membrane microdomains
Zheng, Y.Z. and Foster, L.J.
*J. Proteomics, **72**, 12-22 (2009)*

Many cellular signaling and communication events take place at the plasma membrane and thus the characterization of the plasma membrane proteome has been a hot research area in the hopes of learning more about these processes. Membrane microdomains are large protein and lipid complexes found on the cell surface membrane, able to concentrate or recruit signaling molecules or factors. The first step of any organelle proteomics study is to get a pure and enriched protein sample yet this has always been problematic in membrane proteomics as it is virtually impossible to purify a specific membrane type to homogeneity. In this review, we summarize the biochemical and proteomic approaches that have been

used recently in the isolation and identification of several membrane microdomains and non-typical membrane proteins.

3.1211 Recruitment of protein phosphatase 2A to dorsal ruffles by platelet-derived growth factor in smooth muscle cells: Dephosphorylation of Hsp27
Berrou, E and Brykaert, M.
*Exp. Cell Res., **315**, 836-848 (2009)*

In this study, we investigated the mechanism underlying Hsp27 dephosphorylation in smooth muscle cells. We found that protein phosphatase 2A (PP2A) dephosphorylates Hsp27. In addition, Hsp27 dephosphorylation was regulated by membrane cholesterol content. We showed that PDGF induced a three-fold increase in the proportion of PP2A activity regulated by cholesterol in the Triton-insoluble fraction of cell lysates. Moreover, cholesterol depletion decreased the amount of PP2A recovered in Triton-insoluble fraction. Thus, PDGF might regulate a small pool of PP2A associated with lipid rafts. Isolation of detergent-resistant membrane fragments by Optiprep-gradient density indicated that this pool of PP2A was not associated with caveolae, but was recovered in a higher density fraction (DRM-H) with ganglioside GM1, α-actinin, Hsp27 and p34, a component of Arp2/3 complex. These proteins were also present in dorsal ruffles containing GM1 but not caveolin-1. Phosphorylated Hsp27 levels detected in dorsal ruffles were variable. Cholesterol depletion, which inhibits dorsal ruffle formation, decreased PP2A levels and increased the Hsp27-P to Hsp27 ratio in DRM-H. These findings suggest that Hsp27 is dephosphorylated by PP2A in dorsal ruffles, in non-caveolar lipid raft microdomains. However, similarly to p34, non-phosphorylated Hsp27 is associated to non-raft membrane domains at the leading edge of lamellipodia.

3.1212 HTLV-1 Tax Is a Critical Lipid Raft Modulator That Hijacks IκB Kinases to the Microdomains for Persistent Activation of NF-κB
Huang, J., Ren, T., Guan, H., Jiang, Y. And Cheng, H.
*J.Biol. Chem., **284(10)**, 6208-6217 (2009)*

Upon T cell activation, IκB kinases (IKKs) are transiently recruited to the plasma membrane-associated lipid raft microdomains for activation of NF-κB in promoting T cell proliferation. Retroviral Tax proteins from human T cell leukemia virus type 1 and type 2 (HTLV-1 and -2) are capable of activating IKK, yet only HTLV-1 infection causes T cell leukemia, which correlates with persistent activation of NF-κB induced by Tax1. Here, we show that the Tax proteins exhibit differential modes of IKK activation. The subunits of IKK are constitutively present in lipid rafts in activated forms in HTLV-1-infected T cells that express Tax. Disruption of lipid rafts impairs IκB kinase activation by Tax1. We also show that the cytoplasmic Tax1 protein persistently resides in the Golgi-associated lipid raft microdomains. Tax1 directs lipid raft translocation of IKK through selective interaction with IKKγ and accordingly, depletion of IKKγ impairs Tax1-directed lipid raft recruitment of IKKα and IKKβ. In contrast, Tax2 activates NF-κB in a manner independent of lipid raft recruitment of IKK. These findings indicate that Tax1 actively recruits IKK to the lipid raft microdomains for persistent activation of NF-κB, thereby contributing to HTLV-1 oncogenesis.

3.1213 Proteomic profiling of secretory granules of different T cell subpopulations
Schmidt, H., Gelhaus, C., Nebendahl, M., Leippe, M. and Janssen, O.
*Cell Communication and Signaling, **7 (Suppl 1),** A85 (2009)*

In cytotoxic T lymphocytes and Natural Killer (NK) cells, effector molecules including granzymes, perforin, granulysin and FasL are stored in specialized granules termed secretory lysosomes (SL). These vesicles represent dual-functional organelles that obviously combine degradative and exocytotic properties [1]. We previously established an enrichment protocol to define the proteome of SL from NK cells. We found that the protein content of SL very much depends on the function of a given cell type or clone, best reflected by crucial differences in functionally relevant proteins in transformed NK cell lines [2].
In order to compare the lysosomal content of different T cell subpopulations, we enriched SL from alpha/beta (CD4 or CD8) and gamma/delta (Vdelta 1 or Vdelta2) T cell lines and clones. To this end, the T cell lysates were separated by density gradient centrifugation on Iodixanol gradients. As described before, for the differential proteome analysis we focused on the fraction that contained most FasL, Lamp1 and

Lamp3 (as specific SL or general lysosomal markers) and compared the isolated lysosomal fractions by 2D-DIGE. We found that the protein content of SL of *in vitro* expanded CD4 and CD8 cells as well as Vdelta1 and Vdelta 2 cells is more similar than for example gamma/delta cells compared to alpha/beta cells. A detailed MALDI-based profile of individual SL proteomes based on more than 1000 picked spots from several DIGE experiments will be presented with a focus on the functionally relevant proteins mentioned above. The observed differences might reveal new aspects of population-specific dynamics of activation/maturation and effector function in the T cell compartment.

3.1214 The Comprehensive Native Interactome of a Fully Functional Tagged Prion Protein
Rutishauer, D., Mertz, K.D., Moos, R., Brunner, E., Rülicke, T., Calella, A.M. and Aguzzi, A.
PloSOne, ***4(2)***, *e446 (2009)*

The enumeration of the interaction partners of the cellular prion protein, PrP^{C}, may help clarifying its elusive molecular function. Here we added a carboxy proximal myc epitope tag to PrP^{C}. When expressed in transgenic mice, PrP_{myc} carried a GPI anchor, was targeted to lipid rafts, and was glycosylated similarly to PrP^{C}. PrP_{myc} antagonized the toxicity of truncated PrP, restored prion infectibility of PrP^{C}-deficient mice, and was physically incorporated into PrP^{Sc} aggregates, indicating that it possessed all functional characteristics of genuine PrP^{C}. We then immunopurified myc epitope-containing protein complexes from PrP_{myc} transgenic mouse brains. Gentle differential elution with epitope-mimetic decapeptides, or a scrambled version thereof, yielded 96 specifically released proteins. Quantitative mass spectrometry with isotope-coded tags identified seven proteins which co-eluted equimolarly with PrP^{C} and may represent component of a multiprotein complex. Selected PrP^{C} interactors were validated using independent methods. Several of these proteins appear to exert functions in axomyelinic maintenance.

3.1215 Depletion of Kinesin 5B Affects Lysosomal Distribution and Stability and Induces Peri-Nuclear Accumulation of Autophagosomes in Cancer Cells
Cardoso, C.M.P., Groth-Pedersen, L., Høyer-Hansen, M., Kirkegaard, T., Corcelle, E., Andersen, J.S.-, Jäättelä, M. and Nylandsted, J.
PloSOne, ***4(2)***, *e4424 (2009)*

Background
Enhanced lysosomal trafficking is associated with metastatic cancer. In an attempt to discover cancer relevant lysosomal motor proteins, we compared the lysosomal proteomes from parental MCF-7 breast cancer cells with those from highly invasive MCF-7 cells that express an active form of the ErbB2 (ΔN-ErbB2).
Methodology/Principal Findings
Mass spectrometry analysis identified kinesin heavy chain protein KIF5B as the only microtubule motor associated with the lysosomes in MCF-7 cells, and ectopic ΔN-ErbB2 enhanced its lysosomal association. KIF5B associated with lysosomes also in HeLa cervix carcinoma cells as analyzed by subcellular fractionation. The depletion of KIF5B triggered peripheral aggregations of lysosomes followed by lysosomal destabilization, and cell death in HeLa cells. Lysosomal exocytosis in response to plasma membrane damage as well as fluid phase endocytosis functioned, however, normally in these cells. Both HeLa and MCF-7 cells appeared to express similar levels of the KIF5B isoform but the death phenotype was weaker in KIF5B-depleted MCF-7 cells. Surprisingly, KIF5B depletion inhibited the rapamycin-induced accumulation of autophagosomes in MCF-7 cells. In KIF5B-depleted cells the autophagosomes formed and accumulated in the close proximity to the Golgi apparatus, whereas in the control cells they appeared uniformly distributed in the cytoplasm.
Conclusions/Significance
Our data identify KIF5B as a cancer relevant lysosomal motor protein with additional functions in autophagosome formation.

3.1216 Passage through the Golgi is necessary for Shiga toxin B subunit to reach the endoplasmic reticulum
McKinzie, J., Johannes, L., Taguchi, T. and Sheff, D.
FEBS J., ***276***, *1581-1595 (2009)*

Both Shiga holotoxin and the isolated B subunit, navigate a retrograde pathway from the plasma membrane to the endoplasmic reticulum (ER) of mammalian cells to deliver catalytic A subunits into the cytosol. This route passes through early/recycling endosomes and then through the Golgi. Although passage through the endosomes takes only 30 min, passage through the Golgi is much slower, taking hours. This suggests that Golgi passage is a key step in retrograde traffic. However, there is no empirical data demonstrating that Golgi passage is required for the toxins to enter the ER. In fact, an alternate pathway bypassing the Golgi is utilized by SV40 virus. Here we find that blocking Shiga toxin B access to the entire Golgi with AlF_4^- treatment, temperature block or subcellular surgery prevented Shiga toxin B from reaching the ER. This suggests that there is no direct endosome to ER route available for retrograde traffic. Curiously, when Shiga toxin B was trapped in endosomes, it entered the cytosol directly from the endosomal compartment. Our results suggest that trafficking through the Golgi apparatus is required for Shiga toxin B to reach the ER and that diversion into the Golgi may prevent toxin escape from endosomes into the cytosol.

3.1217 Channel-forming activities of peroxisomal membrane proteins from the yeast *Saccharomyces cerevisiae*

Grunau, S., Mindthoff, S., Rottensteiner, H., Sormuen, R.T., Hitunen, J.K., Erdmann, R. And Antonenkov, V.D.

*FEBS J., **276**, 1698-1708 (2009)*

Highly-purified peroxisomes from the yeast *Saccharomyces cerevisiae* grown on oleic acid were investigated for the presence of channel (pore)-forming proteins in the membrane of these organelles. Solubilized membrane proteins were reconstituted in planar lipid bilayers and their pore-forming activity was studied by means of multiple-channel monitoring or single-channel analysis. Two abundant pore-forming activities were detected with an average conductance of 0.2 and 0.6 nS in 1.0 M KCl, respectively. The high-conductance pore (0.6 nS in 1.0 M KCl) is slightly selective to cations (P_{K+}/P_{Cl-} ~ 1.3) and showed an unusual flickering at elevated (> ±40 mV) holding potentials directed upward relative to the open state of the channel. The data obtained for the properties of the low-conductance pore (0.2 nS in 1.0 M KCl) support the notion that the high-conductance channel represents a cluster of two low-conductance pores. The results lead to conclusion that the yeast peroxisomes contain membrane pore-forming proteins that may aid the transfer of small solutes between the peroxisomal lumen and cytoplasm.

3.1218 Pseudomonas aeruginosa vesicles associate with and are internalized by human lung epithelial cells

Bauman, S.J. and Kuehn, M.J.

*BMC Microbiol., **9**, 26-37 (2009)*

Background

Pseudomonas aeruginosa is the major pathogen associated with chronic and ultimately fatal lung infections in patients with cystic fibrosis (CF). To investigate how *P. aeruginosa*-derived vesicles may contribute to lung disease, we explored their ability to associate with human lung cells.

Results

Purified vesicles associated with lung cells and were internalized in a time- and dose-dependent manner. Vesicles from a CF isolate exhibited a 3- to 4-fold greater association with lung cells than vesicles from the lab strain PAO1. Vesicle internalization was temperature-dependent and was inhibited by hypertonic sucrose and cyclodextrins. Surface-bound vesicles rarely colocalized with clathrin. Internalized vesicles colocalized with the endoplasmic reticulum (ER) marker, TRAPα, as well as with ER-localized pools of cholera toxin and transferrin. CF isolates of *P. aeruginosa* abundantly secrete PaAP (PA2939), an aminopeptidase that associates with the surface of vesicles. Vesicles from a PaAP knockout strain exhibited a 40% decrease in cell association. Likewise, vesicles from PAO1 overexpressing PaAP displayed a significant increase in cell association.

Conclusion

These data reveal that PaAP promotes the association of vesicles with lung cells. Taken together, these results suggest that *P. aeruginosa* vesicles can interact with and be internalized by lung epithelial cells and contribute to the inflammatory response during infection.

3.1219 HIV entry in macrophages is dependent on intact lipid rafts

Carter, G.C., Bernstone, L., Sangani, D., Bee, W.J., Harder, T. and James, W.

*Virology, **386**, 192-202 (2009)*

Macrophages are an important natural target cell for HIV-1, but previous studies of virus entry into these cells are limited, and the involvement of membrane cholesterol and lipid rafts is unknown. Cholesterol disruption of macrophage membranes using four pharmacological agents acting by different mechanisms: methyl-β cyclodextrin, nystatin, filipin complex and Lovastatin, all significantly inhibited productive HIV entry and reverse transcription. The inhibitory effects of these drugs resulted in decreased virus release from infected cells, and could be substantially reversed by the addition of water-soluble cholesterol. The virus bound equally to cholesterol-disrupted cells even though HIV receptor expression levels were significantly reduced. Macrophage CD4 and CCR5 were found to partition with the detergent-resistant membranes with a typical raft-associating protein flotillin-1. HIV particles were observed co-localising with a marker of lipid rafts (CTB-FITC) early post infection. These data suggest that macrophage membrane cholesterol is essential for HIV entry, and implicate lipid raft involvement.

3.1220 Biochemical and functional characterization of the Ror2/BRIb receptor complex
Sammar, M., Sieber, C. and Knaus, P.
Biochem. Biophys. Res. Comm., ***381***, *1-6 (2009)*

Ror2 belongs to the Ror family of receptor tyrosine kinases. Two distinct human disorders result from mutations in Ror2 suggesting a role in cartilage formation, chondrocyte differentiation, and joint formation. We have previously demonstrated functional and physical association of Ror2 with the BMP receptor type Ib (BRIb). The interaction site was mapped to the extracellular CRD domain of Ror2. Here we show specific association with and transphosphorylation by BRIb, but not BMP receptors Ia or II. This association is independent of *N*-glycosylation, excluding the possibility that the interaction is mediated by carbohydrate moieties present in the CRD region of Ror2. The Ror2/BRIb complex proved very stable under high ionic and reducing conditions, yet it appeared sensitive to SDS-treatment. Besides we provide evidence that the Ror2/BRIb complex forms in distinct microdomains at the plasma membrane (DRMs), indicating that Ror2 may interfere with BMP signaling complexes within these membrane domains.

3.1221 Transmembrane Form Agrin-induced Process Formation Requires Lipid Rafts and the Activation of Fyn and MAPK
Ramseger, R., White, R. and Kröger, S.
J. Biol. Chem., ***284(12***, *7697-7705 (2009)*

Overexpression or clustering of the transmembrane form of the extracellular matrix heparan sulfate proteoglycan agrin (TM-agrin) induces the formation of highly dynamic filopodia-like processes on axons and dendrites from central and peripheral nervous system-derived neurons. Here we show that the formation of these processes is paralleled by a partitioning of TM-agrin into lipid rafts, that lipid rafts and transmembrane-agrin colocalize on the processes, that extraction of lipid rafts with methyl-β-cyclodextrin leads to a dose-dependent reduction of process formation, that inhibition of lipid raft synthesis prevents process formation, and that the continuous presence of lipid rafts is required for the maintenance of the processes. Association of TM-agrin with lipid rafts results in the phosphorylation and activation of the Src family kinase Fyn and subsequently in the phosphorylation and activation of MAPK. Inhibition of Fyn or MAPK activation inhibits process formation. These results demonstrate that the formation of filopodia-like processes by TM-agrin is the result of the activation of a complex intracellular signaling cascade, supporting the hypothesis that TM-agrin is a receptor or coreceptor on neurons.

3.1222 Contribution of PIP-5 kinase Iα to raft-based FcγRIIA signaling
Symanska, E., Korzeniowski, M., Raynal, P., Sobota, A. and Kwiatkowska, K.
Exp. Cell Res., ***315***, *981-995 (2009)*

Receptor FcγIIA (FcγRIIA) associates with plasma membrane rafts upon activation to trigger signaling cascades leading to actin polymerization. We examined whether compartmentalization of $PI(4,5)P_2$ and $PI(4,5)P_2$-synthesizing PIP5-kinase Iα to rafts contributes to FcγRIIA signaling. A fraction of PIP5-kinase Iα was detected in raft-originating detergent-resistant membranes (DRM) isolated from U937 monocytes and other cells. The DRM of U937 monocytes contained also a major fraction of $PI(4,5)P_2$. PIP5-kinase Iα bound $PI(4,5)P_2$, and depletion of the lipid displaced PIP5-kinase Iα from the DRM. Activation of FcγRIIA in BHK transfectants led to recruitment of the kinase to the plasma membrane and enrichment of DRM in $PI(4,5)P_2$. Immunofluorescence studies revealed that in resting cells the kinase was associated with the

plasma membrane, cytoplasmic vesicles and the nucleus. After FcγRIIA activation, PIP5-kinase Iα and $PI(4,5)P_2$ co-localized transiently with the activated receptor at distinct cellular locations. Immunoelectron microscopy studies revealed that PIP5-kinase Iα and $PI(4,5)P_2$ were present at the edges of electron-dense assemblies containing activated FcγRIIA in their core. The data suggest that activation of FcγRIIA leads to membrane rafts coalescing into signaling platforms containing PIP5-kinase Iα and $PI(4,5)P_2$.

3.1223 The tyrosine phosphatase SHP-2 controls urokinase-dependent signaling and functions in human vascular smooth muscle cells

Kiyan, J., Haller, H. and Dumler, I.
*Exp. Cell Res., **315**, 1029-1039 (2009)*

The urokinase (uPA)/urokinase receptor (uPAR) multifunctional system is an important mediator of functional behaviour of human vascular smooth muscle cells (VSMC). uPAR associates with platelet-derived growth factor receptor β (PDGFR-β), which serves as a transmembrane adaptor for uPAR in VSMC, to transduce intracellular signaling and initiate functional changes. The precise and rapid propagation of these signaling cascades demands both strict and flexible regulatory mechanisms that remain unexplored. We provide evidence that the tyrosine phosphatase SHP-2 mediates these processes. uPA regulated SHP-2 phosphorylation, catalytic activity, and its co-localization and association with the PDGFR-β. Active PDGFR-β was required for the uPA-induced SHP-2 phosphorylation. uPAR-directed STAT1 pathway was disturbed in cells expressing SHP-2 inactive mutant. Both, cell proliferation and migration were impaired in VSMC with downregulated SHP-2. Elucidating the underlying mechanisms, we found that uPA induced SHP-2 recruitment to lipid rafts. Disruption of rafts abolished uPA-related control of SHP-2 phosphorylation, its association with PDGFR-β and finally the VSMC functional responses. Our results demonstrate that SHP-2 plays an important role in uPA-directed signaling and functional control of human VSMC and suggest that this phosphatase might contribute to the pathogenesis of the uPA-related vascular remodeling.

3.1224 Flagellar membrane localization via association with lipid rafts

Tyler, K.M., Fridberg, A., Toriello, K.M., Olson, C.L., Cieslak, J.A., Hazlett, T.L. and Engman, D.M.
*J. Cell. Sci., **122**, 859-866 (2009)*

The eukaryotic flagellar membrane has a distinct composition from other domains of the plasmalemma. Our work shows that the specialized composition of the trypanosome flagellar membrane reflects increased concentrations of sterols and saturated fatty acids, correlating with direct observation of high liquid order by laurdan fluorescence microscopy. These findings indicate that the trypanosome flagellar membrane possesses high concentrations of lipid rafts: discrete regions of lateral heterogeneity in plasma membranes that serve to sequester and organize specialized protein complexes. Consistent with this, a dually acylated Ca^{2+} sensor that is concentrated in the flagellum is found in detergent-resistant membranes and mislocalizes if the lipid rafts are disrupted. Detergent-extracted cells have discrete membrane patches localized on the surface of the flagellar axoneme, suggestive of intraflagellar transport particles. Together, these results provide biophysical and biochemical evidence to indicate that lipid rafts are enriched in the trypanosome flagellar membrane, providing a unique mechanism for flagellar protein localization and illustrating a novel means by which specialized cellular functions may be partitioned to discrete membrane domains.

3.1225 Identification of a palmitoyl acyltransferase required for protein sorting to the flagellar membrane

Emmer, B.T., Souther, C., Tooriello, K.M., Olson, C.L., Epting, C.L. and Engman, D.M.
*J. Cell Sci., **122**, 867-874 (2009)*

Protein palmitoylation has diverse effects in regulating protein membrane affinity, localization, binding partner interactions, turnover and function. Here, we show that palmitoylation also contributes to the sorting of proteins to the eukaryotic flagellum. African trypanosomes are protozoan pathogens that express a family of unique Ca^{2+}-binding proteins, the calflagins, which undergo N-terminal myristoylation and palmitoylation. The localization of calflagins depends on their acylation status. Myristoylation alone is sufficient for membrane association, but, in the absence of palmitoylation, the calflagins localize to the pellicular (cell body) membrane. Palmitoylation, which is mediated by a specific palmitoyl acyltransferase, is then required for subsequent trafficking of calflagin to the flagellar membrane. Coincident with the redistribution of calflagin from the pellicular to the flagellar membrane is their association with lipid rafts,

which are highly enriched in the flagellar membrane. Screening of candidate palmitoyl acyltranferases identified a single enzyme, TbPAT7, that is necessary for calflagin palmitoylation and flagellar membrane targeting. Our results implicate protein palmitoylation in flagellar trafficking, and demonstrate the conservation and specificity of palmitoyl acyltransferase activity by DHHC-CRD proteins across kingdoms.

3.1226 Can we generate new hypotheses about Dent's disease from gene analysis of a mouse model?
Guggino, S.E.
Exp. Physiol., ***94(2)***, *191-196 (2009)*

In humans, Dent's disease, an X-linked renal tubular disorder, is characterized by low molecular weight proteinuria, aminoaciduria, glycosuria, hyperphosphaturia, hypercalciuria, nephrolithiasis, progressive renal failure and sometimes rickets or osteomalacia. The aetiology of X-linked Dent's disease is established to be caused by mutations of the *CLCN5* gene. The protein product of this gene is the voltage-gated chloride–proton exchanger CLC-5. Previous studies by the Johns Hopkins group (Guggino) and the Hamburg group (Jentsch) have established that the *Clcn5* knockout mouse recapitulates the renal attributes of Dent's disease. In order to understand the changes in kidney function that accompany the knockout of the *Clcn5* gene, we examined gene expression profiles from dissected proximal segment 1 (S1) and segment 2 (S2) tubules of mouse kidneys. Overall, 725 genes are expressed differentially in the proximal tubules of the Dent *Clcn5* knockout mouse model compared with those of control wild-type mice. A major finding is the change in the cholesterol synthesis pathway. Some interesting changes also occur in genes encoding transport proteins. One of these transport proteins, the sodium bile cotransporter gene, *Slc10a2*, has transcripts increased by 17-fold in the *Clcn5* knockout mouse. The Clc-3 protein encoded by *Clcn3*, a chloride–proton exchanger related to Clc-5, has a 1.9-fold increase in transcripts. The Npt2c protein, a proximal tubule sodium phosphate cotransporter encoded by *Slc34a3*, has a 0.6-fold decrease in the number of transcripts. The sodium–proton exchanger-like protein, Nhe10/sperm, encoded by *Slc9a10*, has a 0.5-fold decrease in transcript number. These genes are discussed with regard to the possible physiological outcomes of their transcript or protein changes.

3.1227 The Ubiquitin Ligase c-Cbl Down-Regulates FcγRIIa Activation in Human Neutrophils
Marois, L., Vaillancourt, M., Marois, S., Proulx, S., Pare, G., Rollet-Labelle, E. and Naccache, P.H.
J. Immunol., ***182***, *2374-2384 (2009)*

Little is known about the mechanisms that arrest FcγRIIa signaling in human neutrophils once engaged by immune complexes or opsonized pathogens. In our previous studies, we observed a loss of immunoreactivity of Abs directed against FcγRIIa following its cross-linking. In this study, we report on the mechanisms involved in this event. A stimulated internalization of FcγRIIa leading to the down-regulation of its surface expression was observed by flow cytometry and confocal microscopy. Immunoprecipitation of the receptor showed that FcγRIIa is ubiquitinated after stimulation. MG132 and clasto-lactacystin β-lactone inhibited the loss of immunoreactivity of FcγRIIa, suggesting that this receptor was down-regulated via the proteasomal pathway. The E3 ubiquitin ligase c-Cbl was found to translocate from the cytosol to the plasma membrane following receptor cross-linking. Furthermore, c-Cbl was recruited to the same subset of high-density, detergent-resistant membrane fractions as stimulated FcγRIIa itself. Silencing the expression of c-Cbl by small interfering RNA decreased FcγRIIa ubiquitination and prevented its degradation without affecting the internalisation process. It also prolonged the stimulation of the tyrosine phosphorylation response to the cross-linking of the receptor. We conclude that c-Cbl mediates the ubiquitination of stimulated FcγRIIa and thereby contributes to the termination of FcγRIIa signaling via its proteasomal degradation, thus leading to the down-regulation of neutrophil signalisation and function (phagocytosis) through this receptor.

3.1228 Activation of a nuclear-localized SIPK in tobacco cells challenged by cryptogein, an elicitor of plant defence reactions
Dahan, J., Pichereaux, C., Rossignol, M., Blanc, S., Wendenhenne, D., Pugin, A. and Bourque, S.
Biochem. J., ***418***, *191-200 (2009)*

When a plant cell is challenged by a well-defined stimulus, complex signal transduction pathways are activated to promote the modulation of specific sets of genes and eventually to develop adaptive responses. In this context, protein phosphorylation plays a fundamental role through the activation of multiple protein kinase families. Although the involvement of protein kinases at the plasma membrane and cytosolic levels

are now well-documented, their nuclear counterparts are still poorly investigated. In the field of plant defence reactions, no known study has yet reported the activation of a nuclear protein kinase and/or its nuclear activity in plant cells, although some protein kinases, e.g. MAPK (mitogen-activated protein kinase), are known to be translocated into the nucleus. In the present study, we investigated the ability of cryptogein, a proteinaceous elicitor of tobacco defence reactions, to induce different nuclear protein kinase activities. We found that at least four nuclear protein kinases are activated in response to cryptogein treatment in a time-dependent manner, some of them exhibiting Ca^{2+}-dependent activity. The present study focused on one 47 kDa protein kinase with a Ca^{2+}-independent activity, closely related to the MAPK family. After purification and microsequencing, this protein kinase was formally identified as SIPK (salicyclic acid-induced protein kinase), a biotic and abiotic stress-activated MAPK of tobacco. We also showed that cytosolic activation of SIPK is not sufficient to promote a nuclear SIPK activity, the latter being correlated with cell death. In that way, the present study provides evidence of a functional nuclear MAPK activity involved in response to an elicitor treatment.

3.1229 **Quantification of plasmid DNA copies in the nucleus after lipoplex and polyplex transfection**
Cohen, R.N., van der Aa, M.A.E.M., Macaraeg, N., Lee, A.P. and Szoka Jr., F.C.
J. Controlled Release, ***135***, *166-174 (2009)*

Nuclear uptake of plasmid DNA is one of the many cellular barriers that limit the efficiency of non-viral gene delivery systems. We have determined the number of plasmids that reach the nucleus of a transfected cell using an internally standardized quantitative PCR (qPCR) assay. We isolated nuclei using two different protocols: a density gradient technique and a detergent-based method. The density gradient procedure yielded nuclei with substantially less adhering plasmids on the outside of the nuclei. Using the density gradient protocol we determined that cells transfected with Lipofectamine™ lipoplexes or polyethylenimine polyplexes contained between 75 and 50,000 plasmids/nucleus, depending on the applied plasmid dose. Any increase above 3000 plasmids/nucleus resulted in only marginal increases in transgene expression. Furthermore, lipoplex-delivered plasmids were more efficiently expressed, on the basis of protein expression per plasmid number in the nucleus, than polyplex-delivered plasmids. This indicates that polymer may remain bound to some plasmids in the nucleus. Lastly, by sorting transfected cells into high- and low-expressing sub-populations, we observe that a sub-population of cells contain 3× greater plasmids/nucleus but express nearly 100× more transgene than other cells within a single transfection reaction. Taken together these results suggest the importance of considering the processes downstream from nuclear entry for strategies to improve the efficiency of gene transfer reagents.

Corrigendum to "Quantification of plasmid DNA copies in the nucleus after lipoplex and polyplex transfection"
Cohen, R.N., van der Aa, M.A.E.M., Macaraeg, N., Lee, A.P. and Szoka Jr., F.C.
J. Controlled Release, ***135***, *166-174 (2009)*

The authors regret that there was an error in one of the sentences in Section 2.3 of the above published article. Page 167 column 2, the last sentence at the end of that column should read as follows: Nuclei were recovered from the *30/35%* iodixanol interface by making a hole in the bottom of the ultracentrifuge tube with a 16-gauge needle and collecting fractions into ultracentrifuge tubes.
The authors would like to apologise for any inconvenience this may have caused the readers of the journal.

3.1230 **Expression of reticulon 3 in Alzheimer's disease brain**
Kume, H., Konishis, Y., Murayama, K.S., kametani, F. and Araki, W.
Neuropathol. and Appl. Neurobiol., ***35***, *178-188 (2009)*

Aims:: Reticulon 3 (RTN3), a member of the reticulon family of proteins, interacts with the β-secretase, β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), and inhibits its activity to produce β-amyloid protein. The aim of the present study was to clarify the biological role of RTN3 in the brain and its potential involvement in the neuropathology of Alzheimer's disease (AD).
Methods:: We performed immunohistochemical and biochemical analyses using a specific antibody against RTN3 to investigate the expression and subcellular localization of RTN3 in control and AD brain tissue samples.
Results:: Western blot analysis revealed no significant differences in the RTN3 levels between control and AD brains. Immunohistochemical staining showed that RTN3 immunoreactivity was predominantly localized in pyramidal neurones of the cerebral cortex. The patterns of RTN3 immunostaining were similar in control and AD cerebral cortices, and senile plaques were generally negative for RTN3. Biochemical

subcellular fractionation disclosed that RTN3 colocalized with BACE1 in various fractions, including the endoplasmic reticulum and the Golgi apparatus. Double-immunofluorescence staining additionally indicated that RTN3 was localized in both endoplasmic reticulum and Golgi compartments in neurones. **Conclusions:**: These results show that RTN3 is primarily expressed in pyramidal neurones of the human cerebral cortex and that no clear difference of RTN3 immunoreactivity is observable between control and AD brains. Our data also suggest that there is considerable colocalization of RTN3 with BACE1 at a subcellular level.

3.1231 A high-cholesterol diet increases the association between caveolae and insulin receptors in rat liver
Hahn-Obercyger, M., Graeve, L. and Madar, Z.
J. Lipid Res., ***50***, *98-107 (2009)*

Caveolin-1, a component of caveolae, regulates signaling pathway compartmentalization by interacting with tyrosine (Tyr) kinase receptors and their substrates. Perturbations in caveolae lipid composition have been shown in vitro to displace proteins from lipid microdomains, thereby altering their functionality and subsequent downstream signaling. The role of caveolin-1 in insulin receptor (IR) signaling has been widely investigated in vitro mainly in 3T3-L1 adipocyte cells. However, in vivo experiments investigating this connection in liver tissue have not been carried out. The objective of the present study was to investigate the effects of a high-cholesterol diet on caveolin-1 expression and IR localization and activity in the rat liver. Compared with a standard diet, rats fed with diet rich in cholesterol significantly altered liver caveolae by increasing both caveolin-1 (66%, $P < 0.05$) and caveolin-2 (55%, $P < 0.05$) expression while caveolin-1 mRNA levels were reduced. Concomitantly, a 25% increase in localization of the caveolae-resident signaling protein IR was observed. The distribution of caveolar and noncaveolar phosphorylated IR was unaffected but insulin-induced IR activation was significantly enhanced following consumption of the high-cholesterol diet (120%, $P < 0.001$). However, the downstream molecules IRS-1 and Akt have shown impaired activity in cholesterol-fed rats suggesting insulin resistance condition. Insulin stimulation failed to induce Tyr phosphorylation of caveolin-1 in cholesterol-fed rats. These findings suggest a mechanism by which a high-cholesterol diet altered caveolin-1 expression in vivo accompanied by altered IR localization and activity.

3.1232 Membrane microdomains from early gastrula embryos of medaka, Oryzias latipes , are a platform of E-cadherin- and carbohydrate-mediated cell–cell interactions during epiboly
Adachi, T., Sato, C., Kishi, Y., Totani, K., Murata, T., Usui, T. and Kitajima, K.
Glycoconj. J., ***26***, *285-299 (2009)*

Formation of membrane microdomain is critical for cell migration (epiboly) during gastrulation of medaka fish [Adachi *et al*. (Biochem. Biophys. Res. Commun. 358:848–853, *2007*)]. In this study, we characterized membrane microdomain from gastrula embryos to understand its roles in epiboly. A cell adhesion molecule (E-cadherin), its associated protein (β-catenin), transducer proteins (PLCγ, cSrc), and a cytoskeleton protein (β-actin) were enriched in the membrane microdomain. Le^X-containing glycolipids and glycoproteins (Le^X-gp) were exclusively enriched in the membrane microdomain. Interestingly, the isolated membrane microdomain had the ability to bind to each other in the presence of Ca^{2+}. This membrane microdomain binding was achieved through the E-cadherin homophilic and the Le^X-glycan-mediated interactions. E-cadherin and Le^X-gp were co-localized on the same membrane microdomain, suggesting that these two interactions are operative at the same time. Thus, the membrane microdomain functions as a platform of the E-cadherin- and Le^X-glycan-mediated cell adhesion and signal transduction.

3.1233 Src Family Kinases Accelerate Prolactin Receptor Internalization, Modulating Trafficking and Signaling in Breast Cancer Cells
Piazza, T.M., Lu, J-C., Carver, K.C. and Schuler, L.A.
Mol. Endocrinol., ***23***, *202-212 (2009)*

Despite the growing body of evidence supporting prolactin (PRL) actions in human breast cancer, little is known regarding PRL regulation of its own receptor in these cells. Ligand-initiated endocytosis is a key process in the regulation of receptor availability and signaling cascades that may lead to oncogenic actions. Although exposure to exogenous PRL accelerates degradation of the long isoform of the PRL receptor (lPRLR), neither the signals initiated by PRL that lead to lPRLR internalization and subsequent down-regulation, nor the relationship to downstream pathways are understood in breast cancer cells. In this study, we showed that PRL-induced down-regulation of the lPRLR was reduced by inhibition of src family kinases (SFKs), but not Janus kinase 2, in MCF-7 cells. Inhibition of SFKs also resulted in accumulation of

a PRL-induced PRLR fragment containing the extracellular domain, which appeared to be generated from newly synthesized PRLR. lPRLR was constitutively associated with SFKs in lipid rafts. PRL-induced SFK activation led to recruitment of the guanosine triphosphatase, dynamin-2, to an internalization complex, resulting in endocytosis. Inhibition of endocytosis by small interfering RNA-mediated knockdown of dynamin-2 blocked PRL-induced down-regulation of lPRLR, confirming that internalization is essential for this process. Endocytosis also was required for optimal phosphorylation of ERK1/2 and Akt, but not for Janus kinase 2 or signal transducer and activator of transcription 5, indicating that internalization selectively modulates signaling cascades. Together, these data indicate that SFKs are key mediators of ligand-initiated lPRLR internalization, down-regulation, and signal transduction in breast cancer cells, and underscore the importance of target cell context in receptor trafficking and signal transduction.

3.1234 Downregulation of sodium transporters and NHERF proteins in IBD patients and mouse colitis models: Potential contributors to IBD-associated diarrhea

Sullivan, S., Alex, P., Dassopoulos, T., Zachos, N.C., Iacobuzio-Donahue, C., Donowitz, M., Brant, S.R., Cuffari, C., Harris, M.L., Wu Datta, L., Conklin, L., Chen, Y. and Li, X.
Inflamm. Bowel. Dis., ***15(2)***, *261-274 (2009)*

Background: One of the most common symptoms among patients with inflammatory bowel disease (IBD) is diarrhea, which is thought to be contributed by changes in electrolyte transport associated with intestinal inflammation. This study was designed to test the hypothesis that intestinal Na+-related transporters/channels and their regulatory proteins may be downregulated as a potential contributor to IBD-associated diarrhea.
Methods: SDS-PAGE and Western blotting and/or confocal immunomicroscopy were used to examine the expression of Na+/H+-exchangers 1-3 (NHE1-3), epithelial Na+ channel (ENaC), Na+/K+-ATPase, the intracellular Cl- channel 5 (ClC-5), and NHE3 regulatory factors (NHERF1,2) in ileal and colonic pinch biopsies from IBD patients and noninflammatory controls, as well as from colonic mucosa of dextran sodium sulfate (DSS)- and TNBS-induced acute murine IBD models.
Results: NHE1,3 (but not NHE2), β-ENaC, Na+/K+-ATPase-α, ClC-5, and NHERF1 were all downregulated in sigmoid mucosal biopsies from most cases of active UC and/or CD compared to controls. NHE3 was also decreased in ileal mucosal biopsies of active CD, as well as in ≈50% of sigmoid biopsies from inactive UC or CD. Importantly, similar downregulation of NHE1,3, β-ENaC, and NHERF1,2 was also observed in the mouse colon (but not ileum) of DSS- and TNBS-induced colitis.
Conclusions: IBD-associated diarrhea may be due to a coordinated downregulation of multiple Na+ transporter and related regulatory proteins, including NHE1,3, Na+/K+-ATPase, and ENaC, as well as NHERF1,2, and ClC-5, all of which are involved directly or indirectly in intestinal Na+ absorption.

3.1235 Guanosine Triphosphate Cyclohydrolase I Expression and Enzymatic Activity Are Present in Caveolae of Endothelial Cells

Peterson, T.E., d'Uscio, L.V., Cao, S., Wang, X-L. and Katusic, Z.S.
Hypertension, ***53***, *189-195 (2009)*

Tetrahydrobiopterin is an essential cofactor required for the synthesis of NO. GTP cyclohydrolase I (GTPCH I) is the rate-limiting enzyme for tetrahydrobiopterin production in endothelial cells, yet little is known about the subcellular localization of this enzyme. In this study, we demonstrated that GTPCH I is localized to caveolar membrane microdomains along with caveolin-1 and endothelial NO synthase. GTPCH I activity was detected in isolated caveolar membranes from cultured endothelial cells. Confocal and electron microscopy analyses confirmed GTPCH I colocalization with caveolin-1. Consistent with in vitro studies, GTPCH I activity was evident in isolated caveolar microdomains from lung homogenates of wild-type mice. Importantly, a 2-fold increase in GTPCH I activity was detected in the aortas of caveolin-1–deficient mice, suggesting that caveolin-1 may be involved in the control of GTPCH I enzymatic activity. Indeed, overexpression of caveolin-1 inhibits GTPCH I activity, and tetrahydrobiopterin biosynthesis is activated by the disruption of caveolae structure. These studies demonstrate that GTPCH I is targeted to caveolae microdomains in vascular endothelial cells, and tetrahydrobiopterin production occurs in close proximity to endothelial NO synthase. In addition, our findings provide new insights into the regulation of GTPCH I activity by the caveolar coat protein, caveolin-1.

3.1236 **Isolation of rafts from mouse brain tissue by a detergent-free method**
Persaud-Sawin, D-A., Lightcap, S. and Harry, G.J.
J. Lipid Res., ***50***, *759-767 (2009)*

Membrane rafts are rich in cholesterol and sphingolipids and have specific proteins associated with them. Due to their small size, their identification and isolation have proved to be problematic. Their insolubility in nonionic detergents, such as Triton-X 100, at 4°C has been the most common means of isolation. However, detergent presence can produce artifacts or interfere with ganglioside distribution. The direction is therefore toward the use of detergent-free protocols. We report an optimized method of raft isolation from lipid-rich brain tissue using a detergent-free method. We compared this to Triton-X 100-based isolation along sucrose or **Optiprep**™ gradients using the following endpoints: low protein content, high cholesterol content, presence of Flotillin 1 (Flot1), and absence of transferrin receptor (TfR) proteins. These criteria were met in raft fractions isolated in a detergent-free buffer along a sucrose gradient of 5%/35%/42.5%. The use of **optiprep** gave less consistent results with respect to protein distribution. We demonstrate that clean raft fractions with minimal myelin contamination can be reproducibly obtained in the top three low-density fractions along a sucrose step gradient.

3.1237 **Lipid Raft Segregation Modulates TRPM8 Channel Activity**
Morenilla-Palao, C., Pertusa, M., Meseguer, V., Cabedo, H. and Viana, F.
J. Biol. Chem., ***284(14)***, *9215-9224 (2009)*

Transient receptor potential channels are a family of cation channels involved in diverse cellular functions. Most of these channels are expressed in the nervous system and play a key role in sensory physiology. TRPM8 (transient receptor potential melastatine 8), a member of this family, is activated by cold, cooling substances such menthol and icilin and voltage. Although TRPM8 is a thermosensitive channel highly expressed in cold sensory neurons, the mechanisms underlying its temperature sensitivity are still poorly understood. Here we show that, in sensory neurons, TRPM8 channel is localized in cholesterol-rich specialized membrane domains known as lipid rafts. We also show that, in heterologous expression systems, lipid raft segregation of TRPM8 is favored by glycosylation at the Asn^{934} residue of the polypeptide. In electrophysiological and imaging experiments, using cold and menthol as agonists, we also demonstrate that lipid raft association modulates TRPM8 channel activity. We found that menthol- and cold-mediated responses of TRPM8 are potentiated when the lipid raft association of the channel is prevented. In addition, lipid raft disruption shifts the threshold for TRPM8 activation to a warmer temperature. In view of these data, we suggest a role for lipid rafts in the activity and temperature sensitivity of TRPM8. We propose a model wherein different lipid membrane environments affect the cold sensing properties of TRPM8, modulating the response of cold thermoreceptors.

3.1238 **The amino-terminal region of Atg3 is essential for association with phosphatidylethanolamine in Atg8 lipidation**
Hanada, T., Satomi, Y., Takao, T. and Ohsumi, Y.
FEBS Lett., ***583***, *1078-1083 (2009)*

Autophagy is a bulk degradation process conserved among eukaryotes. In macro-autophagy, autophagosomes sequester cytoplasmic components and deliver their contents to lysosomes/vacuoles. Autophagosome formation requires the conjugation of Atg8, a ubiquitin-like protein, to phosphatidylethanolamine (PE). Here we report that the amino (N)-terminal region of Atg3, an E2-like enzyme for Atg8, plays a crucial role in Atg8–PE conjugation. The conjugating activities of Atg3 mutants lacking the 7 N-terminal amino acid residues or containing a Leu-to-Asp mutation at position 6 were severely impaired both in vivo and in vitro. In addition, the amino-terminal region is critical for interaction with the substrate, PE.

3.1239 **Acute hypertension provokes acute trafficking of distal tubule Na-Cl cotransporter (NCC) to subapical cytoplasmic vesicles**
Lee, D.H., Riquier, A.D.M., Yang, L.E., Leong, P.K.K., Maunsbach, A.B. and McDonough, A.A.
Am. J. Physiol. Renal Physiol., ***296***, *F810-F818 (2009)*

When blood pressure (BP) is elevated above baseline, a pressure natriuresis-diuresis response ensues, critical to volume and BP homeostasis. Distal convoluted tubule Na^+-Cl^- cotransporter (NCC) is regulated by trafficking between the apical plasma membrane (APM) and subapical cytoplasmic vesicles (SCV). We aimed to determine whether NCC trafficking contributes to pressure diuresis by decreasing APM NCC or compensates for increased volume flow to the DCT by increasing APM NCC. BP was raised 50 mmHg (high BP) in rats by arterial constriction for 5 or 20–30 min, provoking a 10-fold diuresis at both times. Kidneys were excised, and NCC subcellular distribution was analyzed by *1*) sorbitol density gradient fractionation and immunoblotting and *2*) immunoelectron microscopy (immuno-EM). NCC distribution did not change after 5-min high BP. After 20–30 min of high BP, 20% of NCC redistributed from low-density, APM-enriched fractions to higher density, endosome-enriched fractions, and, by quantitative immuno-EM, pool size of APM NCC decreased 14% and SCV pool size increased. Because of the time lag of the response, we tested the hypothesis that internalization of NCC was secondary to the decrease in ANG II that accompanies high BP. Clamping ANG II at a nonpressor level by coinfusion of captopril (12 μg/min) and ANG II (20 $ng \cdot kg^{-1} \cdot min^{-1}$) during 30-min high BP reduced diuresis to eightfold and prevented redistribution of NCC from APM- to SCV-enriched fractions. We conclude that DCT NCC may participate in pressure natriuresis-diuresis by retraction out of apical plasma membranes and that the retraction is, at least in part, driven by the fall in ANG II that accompanies acute hypertension.

3.1240 **Phosphotyrosine-dependent *in vitro* reconstitution of recombinant LAT-nucleated multiprotein signalling complexes on liposomes**

Sangani, D., Venien-Bryan, C. and Harder, T.
Mol. Membrane Biol., ***26(2)***, *159-170 (2009)*

Numerous cell surface receptors propagate activation signals to the interior of the cell via tyrosine phosphorylation of transmembrane proteins. This leads to the phosphotyrosine (PiY)-mediated recruitment of cytoplasmic signalling protein complexes which catalyze crucial biochemical signalling reactions. Here we describe the first *in vitro* reconstitution of such PiY-nucleated protein complexes on an artificial lipid membrane. A tyrosine phosphorylated recombinant variant of the transmembrane adaptor protein Linker for Activation of T cells (PiYLAT) was anchored in liposomes. These PiYLAT proteoliposomes specifically recruited cooperative high avidity signalling protein complexes from Jurkat cytosol. Nucleation of signalling protein assemblies readily occurred on PiYLAT liposomes composed of phosphatidylserine, but not on PiYLAT liposomes composed of phosphatidylcholine. Purified recombinant grb2 alone did not stably associate with tyrosine phosphorylated LAT proteoliposomes. However, when grb2 was presented to the PiYLAT proteoliposomes in the context of Jurkat cytosol it was incorporated into multiprotein signalling complexes. Together the data suggest that these reconstituted high-avidity signalling protein complexes represent a cooperative protein network. This novel *in vitro* approach offers a novel technology permitting biochemical, structural, and pharmacological analyses of plasma membrane receptor signalling complexes.

3.1241 **Renal NHE3 and NaPi2 partition into distinct membrane domains**

Riquuier, A.D.M., Lee, D.H., and McDonough, A.A.
Am. J. Physiol. Cell Physiol., ***296***, *C900-C910 (2009)*

Hypertension provokes differential trafficking of the renal proximal tubule Na^+/H^+ exchanger 3 (NHE3) to the base of the apical microvilli and Na^+-P_i cotransporter 2 (NaPi2) to endosomes. The resultant diuresis and natriuresis are key to blood pressure control. We tested the hypothesis that this differential trafficking of NHE3 vs. NaPi2 was associated with partitioning to distinct membrane domains. In anesthetized rats, arterial pressure was increased (104 ± 2 to 142 ± 4 mmHg, 15 min) by arterial constriction and urine output increased 23-fold. Renal membranes were fractionated by cold 1% Triton X-100 extraction then centrifugation through **OptiPrep** flotation gradients. In controls, 84 ± 9% of NHE3 localized to flotillin-enriched lipid raft domains and 69 ± 5% of NaPi2 localized to transferrin receptor-enriched nonrafts. MyosinVI and dipeptidyl peptidase IV, associated with NHE3 regulation, coenriched in lipid rafts with NHE3, while NHE regulatory factor-1 coenriched in nonrafts with NaPi2. Partitioning was not altered by hypertension. Detergent insoluble membranes were pelleted after detergent extraction. NHE3 detergent insolubility decreased as it redistributed from body (80 ± 10% detergent insoluble) to base (75 ± 3%) of the apical microvilli, while NaPi2 partitioned into more insoluble domains as it moved from the microvilli (45 ± 7% detergent insoluble) to endosomes (82 ± 1%). In conclusion, NHE3 and NaPi2, while both localized to apical microvilli, are segregated into domains: NHE3 to lipid rafts and NaPi2 to nonrafts. These domain properties may play a role in the distinct trafficking patterns observed during elevated pressures: NHE3 remains in rafts and settles to the base of the microvilli while NaPi2 is freely endocytosed.

3.1242 Soybean proteomics and its application to functional analysis
Komatsu, S. and Ahsan, N.
*J. Proteomics, **72**, 325-336 (2009)*

Complete genome sequences, which are available for rice and *Arabidopsis*, provide insights into many fundamental aspects of plant biology; they do not, however, address some important aspects of legume biology. Legumes are important for maintenance of human health and as crops for sustainable agriculture. Two model species of legume, *Lotus japonicus* and *Medicago truncatula*, have been the focus of projects on genome sequencing and functional genomics. A project aimed at sequencing the genome of the agricultural legume soybean recently began, but functional genomics studies of this plant are in their infancy, and therefore proteomics approaches could be a powerful tool for functional analysis. In this review, we discuss the strengths and weaknesses of proteomics technologies in soybean biology and we examine the limitations of current techniques.

3.1243 Hydroponics on a chip: Analysis of the Fe deficient Arabidopsis thylakoid membrane proteome
Laganowsky, A., Gomez, S.M., Whitelegge, J.P. and Nishio, J.N.
*J. Proteomics, **72(3)**, 397-415 (2009)*

The model plant *Arabidopsis thaliana* was used to evaluate the thylakoid membrane proteome under Fe-deficient conditions. Plants were cultivated using a novel hydroponic system, called "hydroponics on a chip", which yields highly reproducible plant tissue samples for physiological analyses, and can be easily used for *in vivo* stable isotope labeling. The thylakoid membrane proteome, from intact chloroplasts isolated from Fe-sufficient and Fe-deficient plants grown with hydroponics on a chip, was analyzed using liquid chromatography coupled to mass spectrometry. Intact masses of thylakoid membrane proteins were measured, many for the first time, and several proteins were identified with post-translational modifications that were altered by Fe deficiency; for example, the doubly phosphorylated form of the photosystem II oxygen evolving complex, PSBH, increased under Fe-deficiency. Increased levels of photosystem II protein subunit PSBS were detected in the Fe-deficient samples. Antioxidant enzymes, including ascorbate peroxidase and peroxiredoxin Q, were only detected in the Fe-deficient samples. We present the first biochemical evidence that the two major LHC IIb proteins (LHCB1 and LHCB2) may have significantly different functions in the thylakoid membrane. The study illustrates the utility of intact mass proteomics as an indispensable tool for functional genomics. "Hydroponics on a chip" provides the ability to grow *A. thaliana* under defined conditions that will be useful for systems biology.

3.1244 Advancements in plant proteomics using quantitative mass spectrometry
Oeljeklaus, S., Meyer, H.E. and warscheid, B.
*J. Proteomics, **72(3)**, 545-554 (2009)*

Due to innovative advancements in quantitative MS technologies, proteomics has evolved from taking mere "snapshots" of distinct proteomes in a defined state to monitoring, for instance, changes in abundance, location and/or posttranslational modification(s) of proteins under various conditions, thereby facilitating the functional characterization of proteins in large scale experiments. In plant biology, MS-based quantitative proteomics strategies utilizing stable isotope labeling or label-free methods for protein quantification have only recently been started to find increasing application to comparative and functional proteomics analyses. This review summarizes latest trends and applications in MS-based quantitative plant proteomics and provides insight into different technologies available. In addition, the studies presented here illustrate the enormous potential of quantitative MS for the analysis of important functional aspects with the emphasis on organellar and phosphoproteomics as well as dynamics and turnover of proteins in plants.

3.1245 Long-Distance Delivery of Bacterial Virulence Factors by *Pseudomonas aeruginosa* Outer Membrane Vesicles
Bomberger, J.M., Maceachran, D.P., Coutermarsh, B.A., Ye, S., O'Toole, G.A. and Stanton, B.A.
*PloSPathogens, **5(4)**, e1000382 (2009)*

Bacteria use a variety of secreted virulence factors to manipulate host cells, thereby causing significant morbidity and mortality. We report a mechanism for the long-distance delivery of multiple bacterial virulence factors, simultaneously and directly into the host cell cytoplasm, thus obviating the need for

direct interaction of the pathogen with the host cell to cause cytotoxicity. We show that outer membrane–derived vesicles (OMV) secreted by the opportunistic human pathogen *Pseudomonas aeruginosa* deliver multiple virulence factors, including β-lactamase, alkaline phosphatase, hemolytic phospholipase C, and Cif, directly into the host cytoplasm via fusion of OMV with lipid rafts in the host plasma membrane. These virulence factors enter the cytoplasm of the host cell via N-WASP–mediated actin trafficking, where they rapidly distribute to specific subcellular locations to affect host cell biology. We propose that secreted virulence factors are not released individually as naked proteins into the surrounding milieu where they may randomly contact the surface of the host cell, but instead bacterial derived OMV deliver multiple virulence factors simultaneously and directly into the host cell cytoplasm in a coordinated manner.

3.1246 Characterization of a myristoylated, monomeric HIV Gag protein
Dou, J., Wang, J-J., Chen, X., Li, H., Ding, L. and Spearman, P.
Virology, ***387***, *341-352 (2009)*

The process of HIV assembly requires extensive homomultimerization of the Gag polyprotein on cellular membranes to generate the nascent particle bud. Here we generated a full-length, monomeric Gag polyprotein bearing mutations that eliminated multimerization in living cells as indicated by fluorescence resonance energy transfer (FRET). Monomeric Gag resembled non-myristoylated Gag in its weak membrane binding characteristics and lack of association with detergent-resistant membranes (DRMs or lipid rafts). Monomeric Gag failed to assemble virus-like particles, but was inefficiently rescued into particles by wildtype Gag through the influence of the matrix domain. The subcellular distribution of monomeric Gag was remarkably different than either non-myristoylated Gag or wildtype Gag. Monomeric Gag was found on intracellular membranes and at the plasma membrane, where it highlighted plasma membrane extensions and ruffles. This study indicates that monomeric Gag can traffic to assembly sites in the cell, where it interacts weakly with membranes.

3.1247 Subcellular fractionation of human eosinophils: Isolation of functional specific granules on isoosmotic density gradients
Neves, J.S., Perez, S.A.C., Spencer, L.A., Melo, R.C.N. and Weller, P.F.
J. Immunol., Methods, ***344***, *64-72 (2009)*

Subcellular fractionation has been an important tool in investigating human eosinophil structure and function, including localizing of cytokine/chemokines within granules, investigating granule protein translocation and intracellular transport during eosinophil secretion, and studying secretory mechanisms of granules. The resolution of organelles obtained by subcellular fractionation was improved considerably after the introduction of nonionic iodinated density-gradient metrizamide and Nycodenz media that, unlike sucrose, exhibit relatively low tonicity throughout the gradient. However, the structure and membrane preservation of isolated organelles were still compromised due to the lack of gradient isoosmolarity. This paper describes a detailed protocol of subcellular fractionation of nitrogen cavitated eosinophils on an isoosmotic iodinated density gradient (iodixanol – OptiPrep) and the isolation of well preserved and functional membrane-bound specific granules.

3.1248 Mitochondrial degeneration and not apoptosis is the primary cause of embryonic lethality in ceramide transfer protein mutant mice
Wang, X., Rao, R.P., Kasakowska-Cholody, T., Massod, M.A., Southon, E., Zhang, H., Berthel, C., Nagashim, K., Veenstra, T.K., Tessarollo, L., Acharya, U. and Acharya, J.K.
J. Cell Biol., ***184(1)***, *143-158 (2009)*

Ceramide transfer protein (CERT) functions in the transfer of ceramide from the endoplasmic reticulum (ER) to the Golgi. In this study, we show that CERT is an essential gene for mouse development and embryonic survival and, quite strikingly, is critical for mitochondrial integrity. CERT mutant embryos accumulate ceramide in the ER but also mislocalize ceramide to the mitochondria, compromising their function. Cells in mutant embryos show abnormal dilation of the ER and degenerating mitochondria. These subcellular changes manifest as heart defects and cause severely compromised cardiac function and embryonic death around embryonic day 11.5. In spite of ceramide accumulation, CERT mutant mice do not die as a result of enhanced apoptosis. Instead, cell proliferation is impaired, and expression levels of cell cycle–associated proteins are altered. Individual cells survive, perhaps because cell survival mechanisms are activated. Thus, global compromise of ER and mitochondrial integrity caused by ceramide accumulation in CERT mutant mice primarily affects organogenesis rather than causing cell death via apoptotic pathways.

3.1249 Akt-Mediated Transactivation of the S1P1 Receptor in Caveolin-Enriched Microdomains Regulates Endothelial Barrier Enhancement by Oxidized Phospholipids

Singleton, P.A., Chatchavalvanich, C., Fu, P., Xing, J., Birukova, A.A., Fortune, J.A., Klibanov, A.M., garcia, J.G.N. and Birukov, K.G.

Circ. Res., ***104****, 978-986 (2009)*

Endothelial cell (EC) barrier dysfunction results in increased vascular permeability, leading to increased mass transport across the vessel wall and leukocyte extravasation, the key mechanisms in pathogenesis of tissue inflammation and edema. We have previously demonstrated that OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine) significantly enhances vascular endothelial barrier properties in vitro and in vivo and attenuates endothelial hyperpermeability induced by inflammatory and edemagenic agents via Rac and Cdc42 GTPase dependent mechanisms. These findings suggested potential important therapeutic value of barrier-protective oxidized phospholipids. In this study, we examined involvement of signaling complexes associated with caveolin-enriched microdomains (CEMs) in barrier-protective responses of human pulmonary ECs to OxPAPC. Immunoblotting from OxPAPC-treated ECs revealed OxPAPC-mediated rapid recruitment (5 minutes) to CEMs of the sphingosine 1-phosphate receptor ($S1P_1$), the serine/threonine kinase Akt, and the Rac1 guanine nucleotide exchange factor Tiam1 and phosphorylation of caveolin-1, indicative of signaling activation in CEMs. Abolishing CEM formation (methyl-β-cyclodextrin) blocked OxPAPC-mediated Rac1 activation, cytoskeletal reorganization, and EC barrier enhancement. Silencing (small interfering RNA) Akt expression blocked OxPAPC-mediated $S1P_1$ activation (threonine phosphorylation), whereas silencing $S1P_1$ receptor expression blocked OxPAPC-mediated Tiam1 recruitment to CEMs, Rac1 activation, and EC barrier enhancement. To confirm our in vitro results in an in vivo murine model of acute lung injury with pulmonary vascular hyperpermeability, we observed that selective lung silencing of caveolin-1 or $S1P_1$ receptor expression blocked OxPAPC-mediated protection from ventilator-induced lung injury. Taken together, these results suggest Akt-dependent transactivation of $S1P_1$ within CEMs is important for OxPAPC-mediated cortical actin rearrangement and EC barrier protection.

3.1250 TIP47, a Lipid Cargo Protein Involved in Macrophage Triglyceride Metabolism

Buers, I., Robenek, H., Lorkowski, S., Nitschke, Y., Severs, N.J. and Hofnagel, O.

Arterioscler. Thromb. Vasc. Biol., ***29****, 767-773 (2009)*

***Objective*—** Uptake of lipids by macrophages (MΦ) leads to lipid droplet accumulation and foam cell formation. The PAT family proteins are implicated in lipid droplet formation, but the precise function of the 47-kDa tail interacting protein (TIP47), a member of this family, is poorly defined. The present study was performed to determine the function of TIP47 in MΦ lipid metabolism.

***Methods and Results*—** Freeze-fracture cytochemistry demonstrates that TIP47 is present in the plasma membrane of MΦ and is aggregated into clusters when the cells are incubated with oleate. Suppression of adipophilin levels using siRNA knockdown leads to migration of TIP47 from a cytoplasmic pool to the lipid droplet. Further, reduction of TIP47 decreases triglyceride levels, whereas raising TIP47 levels by expression of EGFP-TIP47 shows the opposite effect.

***Conclusion*—** Our results show that the TIP47 protein levels directly correlate with triglyceride levels. We propose that TIP47 may act as a carrier protein for free fatty acids and in this way participates in conversion of MΦ into foam cells.

The function of TIP47 on MΦ lipid metabolism was investigated. TIP47 protein levels were found to directly correlate with triglyceride levels. From this and other experimental evidence, we propose that TIP47 acts as a carrier protein for free fatty acids and thereby participates in conversion of MΦ into foam cells.

3.1251 Potential abnormalities in iron metabolism in hyperlipidemia patient fibroblasts

Morrison, C., Sauble, E.N., Nguyen, A., La, A., Bach, G. and Linder, M.C.

FASEB J., ***23****, 105.4 (2009)*

Mucolipidosis type IV (MLIV) is an autosomal recessive neurodegenerative disorder that results from a mutation inmucolipin 1, a 580 amino acid non-selective cation channel present on lysosomal membranes. This mutation disruptssorting, transport and/or fusion of endosomes and lysosomes, and subjects suffer from iron deficiency anemia. Toestablish whether lysosomal turnover of endogenous ferritin was disrupted, fibroblasts from normal and MLIVsubjects were pretreated with ^{59}Fe-labeled ferric ammonium citrate (180 μM) for 24h to produce ferritin (Ft) and thenwith desferioxamine to induce Ft turnover in lysosomes

(Kidane et al, Am J Physiol 291: C445, 2006). No defects in Ftturnover and Fe release were observed, as demonstrated with iodixanol density gradients separating cytoplasmic andlysosomes/endosomal Ft and its iron. However, when exogenous (cationized) horse spleen Ft was administered,turnover of this iron was slowed and more was held in lysosomes and endosomes in the case of cells from diseased subjects. Accumulation of cytoplasmic Ft protein (measured by ELISA) was also reduced. We conclude that a reduced rate of processing of iron entering by endocytosis may slow recycling of red cell iron and contribute to the development an iron deficiency-like anemia.

3.1252 Nuclear AT2 receptors mediate angiotensin II-dependent generation of nitric oxide
Gwathmey, T.M., Pendergrass, K.D., Pirro, N.T., Shaltout, H.A., Reid, S.D., Rose, J.C. and Chappell, M.C.
*FASEB J., **23**, 606.9 (2009)*

We recently reported the expression of receptors for the hormone angiotensin II [Ang II] in isolated nuclei of the sheep kidney. The renal cortex contained predominantly AT2 receptors [~70%], while the renal medulla expressed essentially the AT1 subtype [>90%]. The present study examined their functional roles in cortical nuclei of adult sheep. Nuclei were isolated from kidneys of adult (1.5 yr old) sheep by Optiprep density gradient and loaded with the fluorescence dye difluorofluorescein diacetate (DAF) to assess the production of nitric oxide (NO). Ang II (1 nM) significantly increased DAF fluorescence above control (80 +/- 11%; P<0.001; N=4); DAF stimulation was abolished by either the AT2 antagonist PD123319 (1μM, P>0.05 vs. control) or the NO synthase inhibitor L-NAME (1mM, P>0.05 vs. control). Treatment with AT1 receptor antagonist losartan (1μM) did not alter Ang II-induced NO generation (P>0.05 vs. Ang II). Protein analysis of isolated cortical nuclei revealed a prominent band for both eNOS/NOSIII (135kDa) and the principal NO receptor soluble guanylate cyclase-β (sGC, 70kDa). We demonstrate that the nuclear AT2 receptor subtype is functionally linked to NO generation and confirm the expression of eNOS and sGC in sheep nuclei. These data suggest that the nucleus contains the necessary signaling components for NO generation and provide further support of a functional intracellular RAS within the kidney.

3.1253 Mechanisms of iron release from lysosomes
Nguyen, A., Zhao, N., Morrison, C., Gonzales, A., Sauble, E., La, A., Linder, M.C., and Knutson, M.
*FASEB J., **23**, 921.11 (2009)*

Stored cellular Fe is made available and recycled at least partly through lysosomal degradation of cytoplasmic ferritin (Ft) after autophagy, but how Fe returns to the cytoplasm is unknown. Divalent metal transporter 1 (DMT1) is associated not just with transferrin-related endosomes but also with lysosomes, and the latter is true for Zip 8, which might also be an Fe transporter. To begin the determine whether one or both DMT1 and Zip8 might be involved, we first established that Zip 8 transfection into HEK cells (with low endogenous levels) enhanced Fe uptake, determined with 59Fe-citrate. We then knocked down expression of Zip 8 with siRNA in rat hepatoma cells, and studied the accumulation of 59Fe in lysosomes separated from cytoplasmic Ft on iodixanol gradients (Kidane et al, Am J Physiol 291: C445, 2006). To track and move Ft into lysosomes, we pretreated cells with ^{59}Fe-ferric ammonium citrate for 24h then induced Fe depletion with deferoxamine (as previously). Compared with scrambled siRNA, 80-90% knockdown of Zip 8 mRNA was associated with about 35% greater retention of ^{59}Fe in lysosomes. Using confocal microscopy on HepG2 cells, we also determined that Fe depletion rapidly increased colocalization of DMT1 with the lysosomal marker (LAMP2). We conclude that both of these transporters could be involved in the return of lysosomal Fe to the cytoplasm.marker (LAMP2). We conclude that both of these transporters could be involved in the return of lysosomal Fe to the cytoplasm.

3.1254 Protective Role of Endogenous Gangliosides for Lysosomal Pathology in a Cellular Model of Synucleinopathies
Wei, J., Fujita, M., Nakai, M., Waragai, M., Sekigawa, A., Sugama, S., Takenouchi, T., Masliah, E. and Hashimoto, M.
*Am. J. Pathol., **174(5)**, 1891-1909 (2009)*

Gangliosides may be involved in the pathogenesis of Parkinson's disease and related disorders, although the precise mechanisms governing this involvement remain unknown. In this study, we determined whether changes in endogenous ganglioside levels affect lysosomal pathology in a cellular model of synucleinopathy. For this purpose, dementia with Lewy body-linked P123H β-synuclein (β-syn) neuroblastoma cells transfected with α-synuclein were used as a model system because these cells were characterized as having extensive formation of lysosomal inclusions bodies. Treatment of these cells with

D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of glycosyl ceramide synthase, resulted in various features of lysosomal pathology, including compromised lysosomal activity, enhanced lysosomal membrane permeabilization, and increased cytotoxicity. Consistent with these findings, expression levels of lysosomal membrane proteins, ATP13A2 and LAMP-2, were significantly decreased, and electron microscopy demonstrated alterations in the lysosomal membrane structures. Furthermore, the accumulation of both P123H β-syn and α-synuclein proteins was significant in PDMP-treated cells because of the suppressive effect of PDMP on the autophagy pathway. Finally, the detrimental effects of PDMP on lysosomal pathology were significantly ameliorated by the addition of gangliosides to the cultured cells. These data suggest that endogenous gangliosides may play protective roles against the lysosomal pathology of synucleinopathies.

3.1255 Endosomal Nox2 Facilitates Redox-Dependent Induction of NF-κB by TNF-α
Li, Q., Spencer, N.Y., Oakley, F.D., Buettner, G.R. and Engelhardt, J.F.
*Antioxidants & redox Signaling, **11(6)**, 1249-1263 (2009)*

Growing evidence suggests that NADPH oxidase (Nox)-derived reactive oxygen species (ROS) play important roles in regulating cytokine signaling. We have explored how TNF-α induction of Nox-dependent ROS influences NF-κB activation. Cellular stimulation by TNF-α induced NADPH-dependent superoxide production in the endosomal compartment, and this ROS was required for IKK-mediated activation of NF-κB. Inhibiting endocytosis reduced the ability of TNF-α to induce both NADPH-dependent endosomal superoxide and NF-κB, supporting the notion that redox-dependent signaling of the receptor occurs in the endosome. Molecular analyses demonstrated that endosomal H_2O_2 was critical for the recruitment of TRAF2 to the TNFR1/TRADD complex after endocytosis. Studies using both Nox2 siRNA and Nox2-knockout primary fibroblasts indicated that Nox2 was critical for TNF-α–mediated induction of endosomal superoxide. Redox-active endosomes that form after TNF-α or IL-1β induction recruit several common proteins (Rac1, Nox2, $p67^{phox}$, SOD1), while also retaining specificity for ligand-activated receptor effectors. Our studies suggest that TNF-α and IL-1β signaling pathways both can use Nox2 to facilitate redox activation of their respective receptors at the endosomal level by promoting the redox-dependent recruitment of TRAFs. These studies help to explain how cellular compartmentalization of redox signals can be used to direct receptor activation from the plasma membrane.

3.1256 Signaling Components of Redox Active Endosomes: The Redoxosomes
Oakley, F.D., Abbott, D., Li, Q. and Engelhardt, J.F.
*Antioxidants & Redox Signaling, **11(6)**, 1313-1333 (2009)*

Subcellular compartmentalization of reactive oxygen species (ROS) plays a critical role in transmitting cell signals in response to environmental stimuli. In this regard, signals at the plasma membrane have been shown to trigger NADPH oxidase-dependent ROS production within the endosomal compartment and this step can be required for redox-dependent signal transduction. Unique features of redox-active signaling endosomes can include NADPH oxidase complex components (Nox1, Noxo1, Noxa1, Nox2, p47phox, p67phox, and/or Rac1), ROS processing enzymes (SOD1 and/or peroxiredoxins), chloride channels capable of mediating superoxide transport and/or membrane gradients required for Nox activity, and novel redox-dependent sensors that control Nox activity. This review will discuss the cytokine and growth factor receptors that likely mediate signaling through redox-active endosomes, and the common mechanisms whereby they act. Additionally, the review will cover ligand-independent environmental injuries, such as hypoxia/reoxygenation injury, that also appear to facilitate cell signaling through NADPH oxidase at the level of the endosome. We suggest that redox-active endosomes encompass a subset of signaling endosomes that we have termed *redoxosomes*. Redoxosomes are uniquely equipped with redox-processing proteins capable of transmitting ROS signals from the endosome interior to redox-sensitive effectors on the endosomal surface. In this manner, redoxosomes can control redox-dependent effector functions through the spatial and temporal regulation of ROS as second messengers.

3.1257 Isolation of *Saccharomyces Cerevisiae* Mitochondria for Mössbauer, Epr, and Electronic Absorption Spectroscopic Analyses
Lindahl, P.A., Morales, J.G., Miao, R. and Holmes-Hampton, G.
*Methods in Enzymol., **456**, 267-285 (2009)*

Methods are presented to aid in the study of iron metabolism in isolated mitochondria. The “iron-ome” of mitochondria, including the type and concentration of all Fe-containing species in the organelle, is evaluated by integrating the results of four spectroscopic methods, including Mössbauer spectroscopy,

electron paramagnetic resonance, electronic absorption spectroscopy, and inductively coupled plasma mass spectrometry. Although this systems biology approach only allows *groups* of Fe centers to be assessed, rather than individual species, it affords new and useful information. There are many considerations in executing this approach, and this chapter focuses on the practical methods that we have developed for this purpose. First, large quantities of mitochondria are required, and so published isolation methods must be scaled up. Second, mitochondria are isolated under strict anaerobic conditions to allow control of redox state and to protect O_2-sensitive Fe-containing proteins from degradation. Third, the importance of packing mitochondria for both spectroscopic and analytical characterizations is developed. By measuring the volume of packed samples and the percentage of mitochondria contained within that volume, absolute Fe and protein concentrations within the organelle can be obtained. Packing samples into spectroscopy holders also affords maximal signal intensities, which are critical for these studies. Custom inserts designed for this purpose are described. Also described are the designs of a 25-L glass bioreactor, a mechanical cell homogenizer, a device for inserting short EPR tubes into the standard Oxford Instruments EPR cryostat, and a device for transferring samples from Mössbauer holders to EPR tubes while maintaining samples at liquid N_2 temperatures. A brief summary of what we have learned by use of these methods is included.

3.1258 Interruption of Growth Hormone Signaling via SHC and ERK in 3T3-F442A Preadipocytes upon Knockdown of Insulin Receptor Substrate-1

Wang, X., Yang, N., Deng, L., Li, X., Jiang, J., Gan, Y. and Frank, S.J.
Mol. Endocrinol., ***23(4)***, *486-496 (2009)*

Insulin receptor substrate-1 (IRS-1) is a docking protein tyrosine phosphorylated in response to insulin, IGF-1, GH, and other cytokines. IRS-1 has an N-terminal plekstrin homology domain (which facilitates membrane localization), a phosphotyrosine-binding domain [which associates with tyrosine-phosphorylated insulin receptor or IGF-1 receptor (IGF-1R)], and tyrosine residues that, when phosphorylated, bind signaling molecules. The role of IRS-1 in GH signaling is uncertain. We previously reported that IRS-1 and Janus kinase 2 associate independently of tyrosine phosphorylation via IRS-1's N terminus and that IRS-1 reconstitution greatly enhances GH-induced ERK, but not STAT5, activation. We now use GH-responsive 3T3-F442A preadipocytes to study the influence of IRS-1 on GH action. We stably transfected cells with vector only (Control) or a vector encoding IRS-1 short hairpin RNA [knockdown (KD)] and compared representative clones. Immunoblotting confirmed more than 80% knockdown of IRS-1 in KD cells. GH caused characteristic Janus kinase 2 and STAT5 activation in both Control and KD cells, but ERK activation was dramatically reduced in KD cells in GH time course and dose-response experiments. Notably, GH-induced Src homology collagen (SHC) activation and SHC-Grb2 association in KD cells were also markedly diminished compared with Control cells. Subcellular fractionation revealed that IRS-1 in Control cells was largely cytosolic, but the component isolated with plasma membranes was highly enriched in lipid raft membranes (LR). In KD cells, GH-induced ERK activation in the LR fraction was particularly diminished compared with Control cells. These data suggest that LR-enriched IRS-1 contributes substantially to GH-induced ERK activation in LR in 3T3-F442A fibroblasts. Furthermore, our results are consistent with IRS-1 residing upstream of SHC in the GH-induced ERK-signaling pathway.

3.1259 Activation-dependent stabilization of the human thromboxane receptor: role of reactive oxygen species

Wilson, S.J., Cavanagh, C.C., Lesher, A.M., Frey, A.J., Russell, S.E. and Smyth, E.M.
J. Lipid Res., ***50***, *1047-1056 (2009)*

Thromboxane A_2 (TxA_2), the principle product of platelet COX-1-dependent arachidonic acid metabolism, directs multiple pro-atherogenic processes via its receptor, TP. Oxidative challenge offsets TP degradation, a key component in limiting TxA_2's actions. Following TP activation, we observed cellular reactive oxygen species (ROS) generation coincident with increased TP expression. We examined the link between TP-evoked ROS and TP regulation. TP expression was augmented in TPα-transfected cells treated with a TxA_2 analog [1S-1α,2β(5Z),3α(1E,3R*),4α]]-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid (IBOP). This was reduced with a cellular antioxidant, *N*-acetyl cysteine, or two distinct NADPH oxidase inhibitors, diphenyleneiodonium and apocynin. Homologous upregulation of the native TP was also reduced in apocynin-treated aortic smooth muscle cells (ASMCs) and was absent in ASMCs lacking an NADPH oxidase subunit ($p47^{-/-}$). TP transcription was not increased in IBOP-treated cells, indicating a posttranscriptional mechanism. IBOP induced translocation of TPα to the Golgi and reduced degradation of the immature form of the receptor. These data are consistent with a

ROS-dependent mechanism whereby TP activation enhanced TP stability early in posttranscriptional biogenesis. Given the significant role played by TP and ROS in perturbed cardiovascular function, the convergence of TP on ROS-generating pathways for regulation of TxA_2-dependent events may be critical for cardiovascular disease.

3.1260 NHE3 regulatory complexes

Donowitz, M., Mohan, S., Zhu, C.X., Chen, T-E., Lin, R., Cha, B., Zachos, N.C., Murtazina, R., Sarker, R. and Li, X.
J. Exp. Biol., ***212****, 1638-1646 (2009)*

The epithelial brush border Na/H exchanger NHE3 is active under basal conditions and functions as part of neutral NaCl absorption in the intestine and renal proximal tubule, where it accounts for the majority of total Na absorbed. NHE3 is highly regulated. Both stimulation and inhibition occur post-prandially. This digestion related regulation of NHE3 is mimicked by multiple extracellular agonists and intracellular second messengers. The regulation of NHE3 depends on its C-terminal cytoplasmic domain, which acts as a scaffold to bind multiple regulatory proteins and links NHE3 to the cytoskeleton. The cytoskeletal association occurs by both direct binding to ezrin and by indirect binding *via* ezrin binding to the C-terminus of the multi-PDZ domain containing proteins NHERF1 and NHERF2. This is a review of the domain structure of NHE3 and of the scaffolding function and role in the regulation of NHE3 of the NHE3 C-terminal domain.

3.1261 Genetic evidence for the requirement of the endocytic pathway in the uptake of coenzyme Q6 in Saccharomyces cerevisiae

Padilla-Lopez, S., Jimenez-Hildago, M., Martin-Montalvo, A., Clarke, C.F., Navas, P. and Santos-Ocana, C.
Biochim. Biophys. Acta, ***1788****, 1238-1248 (2009)*

Coenzyme Q is an isoprenylated benzoquinone lipid that functions in respiratory electron transport and as a lipid antioxidant. Dietary supplementation with Q is increasingly used as a therapeutic for treatment of mitochondrial and neurodegenerative diseases, yet little is known regarding the mechanism of its uptake. As opposed to other yeast backgrounds, *EG103* strains are unable to import exogenous Q_6 to the mitochondria. Furthermore, the distribution of exogenous Q_6 among endomembranes suggests an impairment of the membrane traffic at the level of the endocytic pathway. This fact was confirmed after the detection of defects in the incorporation of FM4-64 marker and CPY delivery to the vacuole. A similar effect was demonstrated in double mutant strains in Q_6 synthesis and several steps of endocytic process; those cells are unable to uptake exogenous Q_6 to the mitochondria and restore the growth on non-fermentable carbon sources. Additional data about the positive effect of peptone presence for exogenous Q_6 uptake support the hypothesis that Q_6 is transported to mitochondria through an endocytic-based system.

3.1262 Determinants of Secretion and Intracellular Localization of Human Herpesvirus 8 Interleukin-6

Chen, D., Choi, Y.B., Sandford, G. and Nicholas, J.
J. Virol., ***83(13)****, 6874-6882 (2009)*

Human herpesvirus 8 (HHV-8) interleukin-6 (vIL-6) is distinct from human and other cellular IL-6 proteins in that it does not require the nonsignaling α-receptor subunit for the formation of gp130-based signal transducing complexes and also is largely retained intracellularly rather than being secreted. We and others have reported that vIL-6 is retained and is active in the endoplasmic reticulum (ER) compartment, and data from our laboratory have demonstrated that intracellular vIL-6 is functional in the autocrine promotion of proliferation and survival of HHV-8 latently infected primary effusion lymphoma cells. It has also been reported that vIL-6 secretion in gp130-deficient cells can be enhanced by introduced gp130, thereby implicating the signal transducer in vIL-6 trafficking to the cell surface. We examine here the requirements for intracellular retention and localization of vIL-6. Using vIL-6-hIL-6 chimeric and point-mutated vIL-6 proteins, we identified regions and residues of vIL-6 influencing vIL-6 secretion. However, there was no correlation between vIL-6 secretion and gp130 interaction. We found that vIL-6, but not hIL-6, could associate stably with ER-resident chaperone protein calnexin. Glycosylation-dependent interaction of vIL-6 with calnexin correlated with proper protein folding, but there was no direct relationship between vIL-6-calnexin interaction and intracellular retention. While calnexin depletion had little influence on absolute amounts of secreted vIL-6, it led to markedly reduced levels of intracellular cytokine. This was

reversed by gp130 transduction, which had no detectable effect on vIL-6 secretion, but redistributed vIL-6 into ER-distinct locations in calnexin-depleted cells, specifically. Our data reveal that calnexin plays a role in ER localization of vIL-6 and that gp130 promotes ER exit, but not secretion, of the viral cytokine.

3.1263 Psychosine Accumulates in Membrane Microdomains in the Brain of Krabbe Patients, Disrupting the Raft Architecture

White, A.B., Givogri, M.I:, Lopez-Rosas, A., Cao, H., van Breemen, R., Thinakaran, g. and Bongarzozne, E.R.

J. Neurosci., ***29(19)****, 6068-6077 (2009)*

Lipid rafts (LRs) are membrane realms characterized by high concentrations of cholesterol and sphingolipids. Often, they are portrayed as scaffolds on which many different signaling molecules can assemble their cascades. The idea of rafts as scaffolds is garnering significant attention as the consequences of LR disruption have been shown to be manifest in multiple signaling pathways. In this study, LRs in the brain of the twitcher (TWI) mouse, a bona-fide model for infant variants of human globoid cell leukodystrophy or Krabbe disease, were investigated. This mouse has deficient activity of GALC (β-galactosylceramidase) that leads to a progressive accumulation of some galactosyl-sphingolipids in the brain. We hypothesized that the accumulation of psychosine (galactosyl-sphingosine) in the TWI CNS may result in the disruption of rafts in different cell populations such as neurons and oligodendrocytes, both cellular targets during disease. In this communication, we demonstrate that psychosine specifically accumulates in LRs in the TWI brain and sciatic nerve and in samples from brains of human Krabbe patients. It is also shown that this accumulation is accompanied by an increase in cholesterol in these domains and changes in the distribution of the LR markers flotillin-2 and caveolin-1. Finally, we show evidence that this phenomenon may provide a mechanism by which psychosine can exert its known inhibitory effect on protein kinase C. This study provides a previously undescribed biophysical aspect for the mechanism of pathogenesis in Krabbe disease.

3.1264 Cholesterol Regulates the Endoplasmic Reticulum Exit of the Major Membrane Protein P0 Required for Peripheral Myelin Compaction

Saher, G., Quintes, S., Möbius, W., Wehr, M.C., Krämer-Albers, E-M., Bråugger, B. and Nave, K-A.

J. Neurosci., ***29(19)****, 6064-6104 (2009)*

Rapid impulse conduction requires electrical insulation of axons by myelin, a cholesterol-rich extension of the glial cell membrane with a characteristic composition of proteins and lipids. Mutations in several myelin protein genes cause endoplasmic reticulum (ER) retention and disease, presumably attributable to failure of misfolded proteins to pass the ER quality control. Because many myelin proteins partition into cholesterol-rich membrane rafts, their interaction with cholesterol could potentially be part of the ER quality control system. Here, we provide *in vitro* and *in vivo* evidence that the major peripheral myelin protein P0 requires cholesterol for exiting the ER and reaching the myelin compartment. Cholesterol dependency of P0 trafficking in heterologous cells is mediated by a cholesterol recognition/interaction amino acid consensus (CRAC) motif. Mutant mice lacking cholesterol biosynthesis in Schwann cells suffer from severe hypomyelination with numerous uncompacted myelin stretches. This demonstrates that high-level cholesterol coordinates P0 export with myelin membrane synthesis, which is required for the correct stoichiometry of myelin components and for myelin compaction.

3.1265 UBXD4, a UBX-Containing Protein, Regulates the Cell Surface Number and Stability of α3-Containing Nicotinic Acetylcholine Receptors

Rezvani, K., teng, Y., Pan, Y., Dani, J.A., Lindstrom, J., Garcia Gras, E.A., McIntosh, J.M. and De Biasi, M.

J. Neurosci., ***29(21)****, 6883-6896 (2009)*

Adaptor proteins are likely to modulate spatially and temporally the trafficking of a number of membrane proteins, including neuronal nicotinic acetylcholine receptors (nAChRs). A yeast two-hybrid screen identified a novel UBX-containing protein, UBXD4, as one of the cytosolic proteins that interact directly with the α3 and α4 nAChR subunits. The function of UBX-containing proteins is largely unknown. Immunoprecipitation and confocal microscopy confirmed the interaction of UBXD4 with α3-containing nAChRs (α3* nAChRs) expressed in HEK293 cells, PC12 cells, and rat cortical neurons. Overexpression of UBXD4 in differentiated PC12 cells (dPC12) increased nAChR cell surface expression, especially that of the α3β2 subtype. These findings were corroborated by electrophysiology, immunofluorescent staining, and biotinylation of surface receptors. Silencing of UBXD4 led to a significant reduction of α3* nAChRs

in rat cortical neurons and dPC12 cells. Biochemical and immunofluorescence studies of endogenous UBXD4 showed that the protein is located in both the ER and *cis*-Golgi compartments. Our investigations also showed that the α3 subunit is ubiquitinated and that UBXD4 can interfere with its ubiquitination and consequent degradation by the proteasome. Our data suggest that UBXD4 modulates the distribution of α3* nAChRs between specialized intracellular compartments and the plasma membrane. This effect is achieved by controlling the stability of the α3 subunit and, consequently, the number of receptors at the cell surface.

3.1266 Association of FcγRIIa (CD32a) with Lipid Rafts Regulates Ligand Binding Activity
Bournazos, S., Hart, S.P., Chamberlain, L.H., Glennie, M.J. and Dransfield, I.
J. Immunol., ***182***, *8026-8036 (2009)*

Binding of Igs to myeloid cells via FcR is a key event in the control of innate and acquired immunity. FcγRIIa (CD32a) is a receptor for multivalent IgG expressed predominantly by myeloid cells, and its association with microdomains rich in cholesterol and sphingolipids, termed as lipid rafts, has been reported to be essential for efficient signaling. However, for many myeloid cell types, ligand binding to CD32a is suppressed by as yet undefined mechanisms. In this study, we have examined the role of CD32a-lipid raft interactions in the regulation of IgG binding to CD32a. Disruption of lipid raft structure following depletion or sequestration of membrane cholesterol greatly inhibited CD32a-mediated IgG binding. Furthermore, specific CD32a mutants, which show reduced association with lipid rafts (A224S and C241A), displayed decreased levels of IgG binding compared with wild-type CD32a. In contrast, constitutively lipid raft-associated CD32a (GPI-anchored CD32a) exhibited increased capacity for IgG binding compared with the full-length transmembrane CD32a. Our findings clearly suggest a major role for lipid rafts in the regulation of IgG binding and, more specifically, that suppression of CD32a-mediated IgG binding in myeloid cells is achieved by receptor exclusion from lipid raft membrane microdomains.

3.1267 Myosin IIA Associates with NK Cell Lytic Granules to Enable Their Interaction with F-Actin and Function at the Immunological Synapse
Sanborn, K.B., Rak, G.D., maru, S.Y., Demers, K., Difeo, A., Martignetti, J.A., Betts, M.R., Favier, R., Banerjee, P.P. and Orange, J.S.
J. Immunol., ***182***, *6969-6984 (2009)*

NK cell cytotoxicity requires the formation of an actin-rich immunological synapse (IS) with a target cell and the polarization of perforin-containing lytic granules toward the IS. Following the polarization of lytic granules, they traverse through the actin-rich IS to join the NK cell membrane in order for directed secretion of their contents to occur. We examined the role of myosin IIA as a candidate for facilitating this prefinal step in lytic NK cell IS function. Lytic granules in and derived from a human NK cell line, or ex vivo human NK cells, were constitutively associated with myosin IIA. When isolated using density gradients, myosin IIA-associated NK cell lytic granules directly bound to F-actin and the interaction was sensitive to the presence of ATP under conditions of flow. In NK cells from patients with a truncation mutation in myosin IIA, NK cell cytotoxicity, lytic granule penetration into F-actin at the IS, and interaction of isolated granules with F-actin were all decreased. Similarly, inhibition of myosin function also diminished the penetration of lytic granules into F-actin at the IS, as well as the final approach of lytic granules to and their dynamics at the IS. Thus, NK cell lytic granule-associated myosin IIA enables their interaction with actin and final transit through the actin-rich IS to the synaptic membrane, and can be defective in the context of naturally occurring human myosin IIA mutation.

3.1268 Nuclear angiotensin II type 2 (AT2) receptors are functionally linked to nitric oxide production
Gwathmey, T.M., Shaltout, H.A., Pendergrass, K.D., Pirro, N.T., Figueroa, J.P., Rose, J.C., Diz, D.I. and Chappell, M.C.
Am. J. Physiol. Renal Physiol., ***296***, *F1484-F1493 (2009)*

Expression of nuclear angiotensin II type 1 (AT_1) receptors in rat kidney provides further support for the concept of an intracellular renin-angiotensin system. Thus we examined the cellular distribution of renal ANG II receptors in sheep to determine the existence and functional roles of intracellular ANG receptors in higher order species. Receptor binding was performed using the nonselective ANG II antagonist ^{125}I-[Sar^1,Thr^8]-ANG II (^{125}I-sarthran) with the AT_1 antagonist losartan (LOS) or the AT_2 antagonist PD123319 (PD) in isolated nuclei (NUC) and plasma membrane (PM) fractions obtained by differential centrifugation or density gradient separation. In both fetal and adult sheep kidney, PD competed for the majority of cortical NUC (≥70%) and PM (≥80%) sites while LOS competition predominated in medullary NUC (≥

75%) and PM (≥70%). Immunodetection with an AT_2 antibody revealed a single ~42-kDa band in both NUC and PM extracts, suggesting a mature molecular form of the NUC receptor. Autoradiography for receptor subtypes localized AT_2 in the tubulointerstitium, AT_1 in the medulla and vasa recta, and both AT_1 and AT_2 in glomeruli. Loading of NUC with the fluorescent nitric oxide (NO) detector DAF showed increased NO production with ANG II (1 nM), which was abolished by PD and *N*-nitro-L-arginine methyl ester, but not LOS. Our studies demonstrate ANG II receptor subtypes are differentially expressed in ovine kidney, while nuclear AT_2 receptors are functionally linked to NO production. These findings provide further evidence of a functional intracellular renin-angiotensin system within the kidney, which may represent a therapeutic target for the regulation of blood pressure.

3.1269 Caveolin-1 directly interacts with UT-A1 urea transporter: the role of caveolae/lipid rafts in UT-A1 regulation at the cell membrane

Feng, X., Huang, H., Yang, Y., Fröhlich, O., Klein, J.D., Sands, J.M. and Chen, G.
Am. J. Physiol. Renala Physiol., ***296***, *F1514-F1520 (2009)*

The cell plasma membrane contains specialized microdomains called lipid rafts which contain high amounts of sphingolipids and cholesterol. Lipid rafts are involved in a number of membrane protein functions. The urea transporter UT-A1, located in the kidney inner medullary collecting duct (IMCD), is important for urine concentrating ability. In this study, we investigated the possible role of lipid rafts in UT-A1 membrane regulation. Using sucrose gradient cell fractionation, we demonstrated that UT-A1 is concentrated in the caveolae-rich fraction both in stably expressing UT-A1 HEK293 cells and in freshly isolated kidney IMCD suspensions. In these gradients, UT-A1 at the cell plasma membrane is codistributed with caveolin-1, a major component of caveolae. The colocalization of UT-A1 in lipid rafts/caveolae was further confirmed in isolated caveolae from UT-A1-HEK293 cells. The direct association of UT-A1 and caveolin-1 was identified by immunoprecipitation and GST pull-down assay. Examination of internalized UT-A1 in pEGFP-UT-A1 transfected HEK293 cells fluorescent overlap with labeled cholera toxin subunit B, a marker of the caveolae-mediated endocytosis pathway. Disruption of lipid rafts by methyl-β-cyclodextrin or knocking down caveolin-1 by small-interference RNA resulted in UT-A1 cell membrane accumulation. Functionally, overexpression of caveolin-1 in oocytes decreased UT-A1 urea transport activity and UT-A1 cell surface expression. Our results indicate that lipid rafts/caveolae participate in UT-A1 membrane regulation and this effect is mediated via a direct interaction of caveolin-1 with UT-A1.

3.1270 The Organelle Proteome of the DT40 Lymphocyte Cell Line

Hall, S.L., Hester, S., Griffin, J.L., Lilley, K.S. and jackson, A.P.
Mol. Cell. Proteomics, ***8***, *1295-1305 (2009)*

A major challenge in eukaryotic cell biology is to understand the roles of individual proteins and the subcellular compartments in which they reside. Here, we use the localization of organelle proteins by isotope tagging technique to complete the first proteomic analysis of the major organelles of the DT40 lymphocyte cell line. This cell line is emerging as an important research tool because of the ease with which gene knockouts can be generated. We identify 1090 proteins through the analysis of preparations enriched for integral membrane or soluble and peripherally associated proteins and localize 223 proteins to the endoplasmic reticulum, Golgi, lysosome, mitochondrion, or plasma membrane by matching their density gradient distributions to those of known organelle residents. A striking finding is that within the secretory and endocytic pathway a high proportion of proteins are not uniquely localized to a single organelle, emphasizing the dynamic steady-state nature of intracellular compartments in eukaryotic cells.

3.1271 Saccharomyces cerevisiae Na+/H+ Antiporter Nha1p Associates with Lipid Rafts and Requires Sphingolipid for Stable Localization to the Plasma Membrane

Mitsui, K., Hatakeyama, K., Matsushita, M. and Kanazawa, H.
J. Biochem., ***145(6)***, *709-720 (2009)*

The plasma membrane-type Na^+/H^+ antiporter Nha1p from budding yeast plays an important role in intracellular Na^+ and pH homeostasis by mediating the exchange of Na^+ for H^+ across the plasma membrane. However, the mechanism of intracellular targeting of Nha1p to the plasma membrane remains unknown. Here, we found that Nha1p exists predominantly in detergent-resistant membrane fractions (DRMs) following density gradient centrifugation. When ergosterol was extracted from membranes, Nha1p was transferred to a detergent-soluble fraction, suggesting that Nha1p associates with ergosterol-containing DRMs, also known as lipid rafts. Density gradient centrifugation of cell extracts of yeast mutants that were defective in different stages of the secretory pathway revealed that, unlike previously

identified raft proteins, the association of Nha1p with DRMs occurs mainly at the plasma membrane. In *lcb1-100* cells, which are temperature-sensitive for sphingolipid synthesis, newly synthesized Nha1p failed to localize to the plasma membrane at the non-permissive temperature. Rather, Nha1p was distributed in an intracellular punctate pattern. The addition of phytosphingosine or the inhibition of endocytosis in *lcb1-100* cells restored the targeting of Nha1p to the plasma membrane. The results of the current study suggest that sphingolipids are required for the stable localization of Nha1p to the plasma membrane.

3.1272 A genome-wide screen for genes affecting eisosomes reveals Nce102 function in sphingolipid signaling

Frölich, F., Moreira, K., Aguilar, P.S., Hubner, N.C., Mann, M., Walter, P. and Walther, T.C.
J. Cell Biol., ***185(7)***, *1227-1242 (2009)*

The protein and lipid composition of eukaryotic plasma membranes is highly dynamic and regulated according to need. The sphingolipid-responsive Pkh kinases are candidates for mediating parts of this regulation, as they affect a diverse set of plasma membrane functions, such as cortical actin patch organization, efficient endocytosis, and eisosome assembly. Eisosomes are large protein complexes underlying the plasma membrane and help to sort a group of membrane proteins into distinct domains. In this study, we identify Nce102 in a genome-wide screen for genes involved in eisosome organization and Pkh kinase signaling. Nce102 accumulates in membrane domains at eisosomes where Pkh kinases also localize. The relative abundance of Nce102 in these domains compared with the rest of the plasma membrane is dynamically regulated by sphingolipids. Furthermore, Nce102 inhibits Pkh kinase signaling and is required for plasma membrane organization. Therefore, Nce102 might act as a sensor of sphingolipids that regulates plasma membrane function.

3.1273 Alterations of Mitochondrial Enzymes Contribute to Cardiac Hypertrophy before Hypertension Development in Spontaneously Hypertensive Rats

Meng, C., Jin, X., Xia, L., Shen, S-M., Wang, X-L., Cai, J., Chen, G-Q., Wang, L-S., and Fang, N-Y.
J. Proteome Res., ***8***, *2463-2475 (2009)*

Mitochondrial dysfunction is recently thought to be tightly associated with the development of cardiac hypertrophy as well as hypertension. However, the detailed molecular events in mitochondria at early stages of hypertrophic pathogenesis are still unclear. Applying two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) combined with MALDI-TOF/TOF tandem mass spectrometry, here we identified the changed mitochondrial proteins of left ventricular mitochondria in prehypertensive/hypertensive stages of cardiac hypertrophy through comparing spontaneously hypertensive rats (SHR) and the age-matched normotensive Wistar Kyoto (WKY) rats. The results revealed that in the hypertrophic left ventricle of SHR as early as 4 weeks old with normal blood pressure, 33 mitochondrial protein spots presented significant alterations, with 17 down-regulated and 16 up-regulated. Such alterations were much greater than those in 20-week-old SHR with elevated blood pressure. Of the total alterations, the expression of two mitochondrial enzymes, trifunctional enzyme alpha subunit (Hadha) and NADH dehydrogenase 1 alpha subcomplex 10 (Ndufa10), were found to have special expression modification patterns in SHR strain. These data would provide new clues to investigate the potential contribution of mitochondrial dysfunction to the development of cardiac hypertrophy.

3.1274 The enterocyte microvillus is a vesicle-generating organelle

McConnell, R.E., Higginbotham, J.N., Shifrin, D.A., Tabb, D.L., Coffey, R.J. and Tyska, M.J.
J. Cell Biol., ***185(7)***, *1285-1298 (2009)*

For decades, enterocyte brush border microvilli have been viewed as passive cytoskeletal scaffolds that serve to increase apical membrane surface area. However, recent studies revealed that in the in vitro context of isolated brush borders, myosin-1a (myo1a) powers the sliding of microvillar membrane along core actin bundles. This activity also leads to the shedding of small vesicles from microvillar tips, suggesting that microvilli may function as vesicle-generating organelles in vivo. In this study, we present data in support of this hypothesis, showing that enterocyte microvilli release unilamellar vesicles into the intestinal lumen; these vesicles retain the right side out orientation of microvillar membrane, contain catalytically active brush border enzymes, and are specifically enriched in intestinal alkaline phosphatase. Moreover, myo1a knockout mice demonstrate striking perturbations in vesicle production, clearly implicating this motor in the in vivo regulation of this novel activity. In combination, these data show that microvilli function as vesicle-generating organelles, which enable enterocytes to deploy catalytic activities into the intestinal lumen.

3.1275 High-Resolution Fractionation of Signaling Endosomes Containing Different Receptors
McCaffrey, G., Welker, J., Scott, J., van der Salm, L. and Grimes, M.L.
*Traffic, **10(7)**, 938-950 (2009)*

Receptor endocytosis is regulated by ligand binding, and receptors may signal after endocytosis in signaling endosomes. We hypothesized that signaling endosomes containing different types of receptors may be distinct from one another and have different physical characteristics. To test this hypothesis, we developed a high-resolution organelle fractionation method based on mass and density, optimized to resolve endosomes from other organelles. Three different types of receptors undergoing ligand-induced endocytosis were localized predominately in endosomes that were resolved from one another using this method. Endosomes containing activated receptor tyrosine kinases (RTKs), TrkA and EGFR, were similar to one another. Endosomes containing $p75^{NTR}$ (in the tumor necrosis receptor superfamily) and PAC1 (a G-protein-coupled receptor) were distinct from each other and from RTK endosomes. Receptor-specific endosomes may direct the intracellular location and duration of signal transduction pathways to dictate response to signals and determine cell fate.

3.1276 Applications of proteomics in the study of inflammatory bowel diseases: Current status and future directions with available technologies
Alex, P., Gucek, M. and Li, X.
*Inflamm. Bowel Dis., **15(4)**. 616-629 (2009)*

Inflammatory bowel diseases (IBD) are chronic, heterogeneous, and multifactorial intestinal inflammatory disorders. Major challenges in IBD research include identification of major pathogenic alterations of genes/proteins as well as effective biomarkers for early diagnosis, prognosis, and prediction of therapeutic response. Since proteins govern cellular structure and biological function, a wide selection of proteomic approaches enables effective characterization of IBD pathogenesis by investigating the dynamic nature of protein expression, cellular and subcellular distribution, posttranslational modifications, and interactions at both the cellular and subcellular levels. The aims of this review are to 1) highlight the current status of proteomic studies of IBD, and 2) introduce the available and emerging proteomic technologies that have potential applications in the study of IBD. These technologies include various mass spectrometry technologies, quantitative proteomics (2D-PAGE, ICAT, SILAC, iTRAQ), protein/antibody arrays, and multi-epitope-ligand cartography. This review also presents information and methodologies, from sample selection and enrichment to protein identification, that are not only essential but also particularly relevant to IBD research. The potential future application of these technologies is expected to have a significant impact on the discovery of novel biomarkers and key pathogenic factors for IBD.

3.1277 A Single Conserved Leucine Residue on the First Intracellular Loop Regulates ER Export of G Protein-Coupled Receptors
Duvernay, M.T., Dong, C., Zhang, X., Robitaille, M., Hebert, T.E. and Wu, G.
*Traffic, **10**, 552-566 (2009)*

The intrinsic structural determinants for export trafficking of G protein-coupled receptors (GPCRs) have been mainly identified in the termini of the receptors. In this report, we determined the role of the first intracellular loop (ICL1) in the transport from the endoplasmic reticulum (ER) to the cell surface of GPCRs. The [alpha]2B-adrenergic receptor (AR) mutant lacking the ICL1 is unable to traffic to the cell surface and to initiate signaling measured as ERK1/2 activation. Mutagenesis studies identify a single Leu48 residue in the ICL1 modulates [alpha]2B-AR export from the ER. The ER export function of the Leu48 residue can be substituted by Phe, but not Ile, Val, Tyr and Trp, and is unlikely involved in correct folding or dimerization of [alpha]2B-AR in the ER. Importantly, the isolated Leu residue is remarkably conserved in the center of the ICL1s among the family A GPCRs and is also required for the export to the cell surface of [beta]2-AR, [alpha]1B-AR and angiotensin II type 1 receptor. These data indicate a crucial role for a single Leu residue within the ICL1 in ER export of GPCRs.

3.1278 Cytoplasmic prion protein induces forebrain neurotoxicity
Wang, X., Bowers, S.L., Wang, F., Pu, X-a., Nelson, R.J. and Ma, J.
*Biochim. Biophys. Acta, **1792**, 555-563 (82009)*

The prion protein (PrP) is essential for the pathogenesis of prion disease. PrP has been detected in the cytosol of neurons and transgenic mice expressing PrP in the cytosol (cyPrP) under a pan-neuronal promoter developed rapid cerebellar granule neuron degeneration. Yet, it remains unclear whether cyPrP is capable to cause toxicity in other neuronal populations. Here, we report that transgenic mice expressing cyPrP in the forebrain neurons developed behavioral abnormalities including clasping and hyperactivity. These mice had reduced thickness in cortex and developed astrogliosis in hippocampal and cortical regions. Moreover, cyPrP in these mice was recognized by the A11 anti-oligomer antibody and was associated with the hydrophobic lipid core of membranes, indicating that cyPrP oligomer caused membrane perturbation contributes to cyPrP neurotoxicity. Together, our results clearly revealed that cyPrP is able to cause toxicity in different neuronal populations, supporting a role of cyPrP in PrP-mediated neurodegenerative disorders.

3.1279 Interactions of DNA with Biofilm-Derived Membrane Vesicles
Schooling, S.R., Hubley, A. and Beveridge, T.J.
J. Bacteriol., ***191(13)***, *4097-4102 (2009)*

The biofilm matrix contributes to the chemistry, structure, and function of biofilms. Biofilm-derived membrane vesicles (MVs) and DNA, both matrix components, demonstrated concentration-, pH-, and cation-dependent interactions. Furthermore, MV-DNA association influenced MV surface properties. This bears consequences for the reactivity and availability for interaction of matrix polymers and other constituents.

3.1280 Derlin-dependent accumulation of integral membrane proteins at cell surfaces
Schaheen, B., Dang, H. and Fares, H.
J. Cell Sci., ***122***, *2228-2239 (2009)*

Quality-control mechanisms of protein folding of transmembrane and secreted proteins is mediated by endoplasmic-reticulum-associated degradation (ERAD), which is used to detect and to degrade misfolded proteins in the ER. The ERAD machinery consists of chaperones, transmembrane proteins and ubiquitin-associated enzymes that detect, modify, and retro-translocate the misfolded proteins to the cytoplasm for degradation by the proteasome. In contrast to ERAD, little is known about the fates of integral membrane and secreted proteins that become misfolded at the plasma membrane or in the extracellular space. Derlin proteins are a family of proteins that are conserved in all eukaryotes, where they function in ERAD. Here, we show that loss of Derlin function in *Caenorhabditis elegans* and in mouse macrophages results in the accumulation of integral membrane proteins at the plasma membrane. Induction of LDL receptor misfolding at the plasma membrane results in a sharp decrease in its half-life, which can be rescued by proteasomal inhibitors or by reduction of Derlin-1 levels. We also show that Derlin proteins localize to endosomes as well as to the ER. Our data are consistent with a model where Derlin proteins function in a spatially segregated quality control pathway that is used for the recognition and degradation of transmembrane proteins that become misfolded at the plasma membrane and/or in endosomes.

3.1281 Functional implications of the influence of ABCA1 on lipid microenvironment at the plasma membrane: a biophysical study
Zarubica, A., Plazzo, A.P., Stöckl, M., Trombik, T., Hamon, Y., Müller, P., pomorski, T., Herrmann, A. and Chimini, G.
FASEB J., ***23***, *1775-1785 (2009)*

The ABCA1 transporter orchestrates cellular lipid homeostasis by promoting the release of cholesterol to plasmatic acceptors. The molecular mechanism is, however, unknown. We report here on the biophysical analysis in living HeLa cells of the ABCA1 lipid microenvironment at the plasma membrane. The modifications of membrane attributes induced by ABCA1 were assessed at both the outer and inner leaflet by monitoring either the lifetime of membrane inserted fluorescent lipid analogues by fluorescence lifetime imaging microscopy (FLIM) or, respectively, the membrane translocation of cationic sensors. Analysis of the partitioning of dedicated probes in plasma membrane blebs vesiculated from these cells allowed visualization of ABCA1 partitioning into the liquid disordered-like phase and corroborated the idea that ABCA1 destabilizes the lipid arrangement at the membrane. Specificity was demonstrated by comparison with cells expressing an inactive transporter. The physiological relevance of these modifications was finally demonstrated by the reduced membrane mobility and function of transferrin receptors under the influence of an active ABCA1. Collectively, these data assess that the control of both transversal and lateral lipid distribution at the membrane is the primary function of ABCA1 and positions the effluxes of

cholesterol from cell membranes downstream to the redistribution of the sterol into readily extractable membrane pools.

3.1282 **In vitro import of peroxisome-targeting signal type 2 (PTS2) receptor Pex7p into peroxisomes**
Miyata, N., Hosoi, K-i., Mukai, S. and Fujiki, Y.
*Biochim. Biophys. Acta, **1793**, 860-870 (2009)*

Pex7p, the peroxisome-targeting signal type 2 (PTS2) receptor, transports PTS2 proteins to peroxisomes from the cytosol. We here established a cell-free Pex7p translocation system. In assays using post-nuclear supernatant fractions each from wild-type CHO-K1 and *pex7* ZPG207 cells, ^{35}S-labeled Pex7p was imported into peroxisomes. ^{35}S-Pex7p import was also evident using rat liver peroxisomes. ^{35}S-Pex7p was not imported into peroxisomal remnants from a *pex5* ZPG231 defective in PTS2 import and *pex2* Z65. When the import of ^{35}S-Pex5pL was inhibited with an excess amount of recombinant Pex5pS, ^{35}S-Pex7p import was concomitantly abrogated, suggesting that Pex5pL was a transporter for Pex7p, unlike a yeast cochaperone, Pex18p. ^{35}S-Pex7p as well as ^{35}S-Pex5p was imported in an ATP-independent manner, whilst the import of PTS1 and PTS2 cargo-proteins was ATP-dependent. Thereby, ATP-independent import of Pex7p implicated that Pex5p export requiring ATP hydrolysis is not a limiting step for its cargo recruitment to peroxisomes. PTS1 protein import was indeed insensitive to N-ethylmaleimide, whereas Pex5p export was N-ethylmaleimide-sensitive. Taken together, the cargo-protein translocation through peroxisomal membrane more likely involves another ATP-requiring step in addition to the Pex5p export. Moreover, upon concurrent import into peroxisomes, ^{35}S-Pex5pL and ^{35}S-Pex7p were detected at mutually distinct ratios in the immunoprecipitates each of the import machinery peroxins including Pex14p, Pex13p, and Pex2p, hence suggesting that Pex7p as well as Pex5p translocated from the initial docking complex to RING complex on peroxisomes.

3.1283 **Sphingosine Facilitates SNARE Complex Assembly and Activates Synaptic Vesicle Exocytosis**
Darios, F. et al
*Neuron, **62**, 683-694 (2009)*

Synaptic vesicles loaded with neurotransmitters fuse with the plasma membrane to release their content into the extracellular space, thereby allowing neuronal communication. The membrane fusion process is mediated by a conserved set of SNARE proteins: vesicular synaptobrevin and plasma membrane syntaxin and SNAP-25. Recent data suggest that the fusion process may be subject to regulation by local lipid metabolism. Here, we have performed a screen of lipid compounds to identify positive regulators of vesicular synaptobrevin. We show that sphingosine, a releasable backbone of sphingolipids, activates synaptobrevin in synaptic vesicles to form the SNARE complex implicated in membrane fusion. Consistent with the role of synaptobrevin in vesicle fusion, sphingosine upregulated exocytosis in isolated nerve terminals, neuromuscular junctions, neuroendocrine cells and hippocampal neurons, but not in neurons obtained from *synaptobrevin-2* knockout mice. Further mechanistic insights suggest that sphingosine acts on the synaptobrevin/phospholipid interface, defining a novel function for this important lipid regulator.

3.1284 **Taking the Scenic Route: Biosynthetic Traffic to the Plasma Membrane in Polarized Epithelial Cells**
Fölsch, H., Mattila, P.E. and Weisz, O.A.
*Traffic, **10(8)**, 972-981 (2009)*

The maintenance of epithelial cell function requires the establishment and continuous renewal of differentiated apical and basolateral plasma membrane domains with distinct lipid and protein compositions. Newly synthesized proteins destined for either surface domain are processed along the biosynthetic pathway and segregated into distinct subsets of transport carriers emanating from the ***trans***-Golgi network. Recent studies have illuminated additional complexities in the subsequent delivery of these proteins to the cell surface. In particular, multiple routes to the apical and basolateral cell surfaces have been uncovered, and many of these involve indirect passage through endocytic compartments. This review summarizes our current understanding of these routes and discusses open issues that remain to be clarified.

3.1285 **SHMT1 and SHMT2 Are Functionally Redundant in Nuclear De novo Thymidylate Biosynthesis**
Anderson, D.D. and Stover, P.J.
*PloSOne, **4(6)**, e5839 (2009)*

The three enzymes that constitute the *de novo* thymidylate synthesis pathway in mammals, cytoplasmic serine hydroxymethyltransferase (SHMT1), thymidylate synthase (TYMS) and dihydrofolate reductase (DHFR) undergo sumoylation and nuclear import during S-phase. In this study, we demonstrate that purified intact mouse liver nuclei convert dUMP to dTMP in the presence of NADPH and serine. Neither nuclear extracts nor intact nuclei exposed to aminomethylphosphonate, a SHMT inhibitor, exhibit thymidylate synthesis activity. Nuclei isolated from *Shmt1*$^{-/-}$ mouse livers retained 25% of thymidylate synthesis activity exhibited by nuclei isolated from wild type mice. This residual activity was due to the presence of a cytoplasmic/nuclear isozyme of SHMT encoded by *Shmt2*. *Shmt2* is shown to encode two transcripts, one which encodes a protein that localizes exclusively to the mitochondria (SHMT2), and a second transcript that lacks exon 1 and encodes a protein that localizes to the cytoplasm and nucleus during S-phase (SHMT2α). The ability of *Shmt2* to encode a cytoplasmic isozyme of SHMT may account for the viability of *Shmt1*$^{-/-}$ mice and provide redundancy that permitted the expansion of the human *SHMT1* L474F polymorphism that impairs SHMT1 sumoylation and nuclear translocation.

3.1286 Lipid Rafts and Clathrin Cooperate in the Internalization of PrPC in Epithelial FRT Cells

Sernataro, D., Caputo, A., Casanova, P., Puri, C., Paladino, S., Tovodar, S.S., Campana, V., Tacchetti, C. and Zurzolo, C.
PloSOne, ***4(6)***, *e5829 (2009)*

Background
The cellular prion protein (PrPC) plays a key role in the pathogenesis of Transmissible Spongiform Encephalopathies in which the protein undergoes post-translational conversion to the infectious form (PrPSc). Although endocytosis appears to be required for this conversion, the mechanism of PrPC internalization is still debated, as caveolae/raft- and clathrin-dependent processes have all been reported to be involved.
Methodology/Principal Findings
We have investigated the mechanism of PrPC endocytosis in Fischer Rat Thyroid (FRT) cells, which lack caveolin-1 (cav-1) and caveolae, and in FRT/cav-1 cells which form functional caveolae. We show that PrPC internalization requires activated Cdc-42 and is sensitive to cholesterol depletion but not to cav-1 expression suggesting a role for rafts but not for caveolae in PrPC endocytosis. PrPC internalization is also affected by knock down of clathrin and by the expression of dominant negative Eps15 and Dynamin 2 mutants, indicating the involvement of a clathrin-dependent pathway. Notably, PrPC co-immunoprecipitates with clathrin and remains associated with detergent-insoluble microdomains during internalization thus indicating that PrPC can enter the cell via multiple pathways and that rafts and clathrin cooperate in its internalization.
Conclusions/Significance
These findings are of particular interest if we consider that the internalization route/s undertaken by PrPC can be crucial for the ability of different prion strains to infect and to replicate in different cell lines.

3.1287 In Vivo Generation of Neurotoxic Prion Protein: Role for Hsp70 in Accumulation of Misfolded Isoforms

Fernandez-Funez, P., Casas-Tinto, S., Zhang, Y., Gomez-Velazquez, M., Morales-Garza, M.A., Cepeda-Nieto, A.C., Castilla, J., Soto, C., Rincon-Limas, D.E.
PloSGenetics, ***5(6)***, *e1000507 (2009)*

Prion diseases are incurable neurodegenerative disorders in which the normal cellular prion protein (PrP^{C}) converts into a misfolded isoform (PrP^{Sc}) with unique biochemical and structural properties that correlate with disease. In humans, prion disorders, such as Creutzfeldt-Jakob disease, present typically with a sporadic origin, where unknown mechanisms lead to the spontaneous misfolding and deposition of wild type PrP. To shed light on how wild-type PrP undergoes conformational changes and which are the cellular components involved in this process, we analyzed the dynamics of wild-type PrP from hamster in transgenic flies. In young flies, PrP demonstrates properties of the benign PrP^{C}; in older flies, PrP misfolds, acquires biochemical and structural properties of PrP^{Sc}, and induces spongiform degeneration of brain neurons. Aged flies accumulate insoluble PrP that resists high concentrations of denaturing agents and contains PrP^{Sc}-specific conformational epitopes. In contrast to PrP^{Sc} from mammals, PrP is proteinase-sensitive in flies. Thus, wild-type PrP rapidly converts in vivo into a neurotoxic, protease-sensitive isoform distinct from prototypical PrP^{Sc}. Next, we investigated the role of molecular chaperones in PrP misfolding in vivo. Remarkably, Hsp70 prevents the accumulation of PrP^{Sc}-like conformers and protects against PrP-dependent neurodegeneration. This protective activity involves the direct interaction between Hsp70 and PrP, which may occur in active membrane microdomains such as lipid rafts, where we detected Hsp70.

These results highlight the ability of wild-type PrP to spontaneously convert in vivo into a protease-sensitive isoform that is neurotoxic, supporting the idea that protease-resistant PrP^{Sc} is not required for pathology. Moreover, we identify a new role for Hsp70 in the accumulation of misfolded PrP. Overall, we provide new insight into the mechanisms of spontaneous accumulation of neurotoxic PrP and uncover the potential therapeutic role of Hsp70 in treating these devastating disorders.

3.1288 A novel sorting strategy of trichosanthin for hijacking human immunodeficiency virus type 1
Zhao, W-L., Zhang, F., Feng, D., Shan, J.W., Chen, S. and Sui, S-F.
*Biochem. Biophys. Res. Comm.,**384**, 347-351 (2009)*

Trichosanthin (TCS) is a type I ribosome-inactivating protein that plays dual role of plant toxin and anti-viral peptide. The sorting mechanism of such an exogenous protein is in long pursuit. Here, we examined TCS trafficking in cells expressing the HIV-1 scaffold protein Gag, and we found that TCS preferentially targets the Gag budding sites at plasma membrane or late endosomes depending on cell types. Lipid raft membrane but not the Gag protein mediates the association of TCS with viral components. After Gag budding, TCS is then released in association with the virus-like particles to generate TCS-enriched virions. The resulting TCS-enriched HIV-1 exhibits severely impaired infectivity. Overall, the observations indicate the existence of a unique and elaborate sorting strategy for hijacking HIV-1.

3.1289 Vesicle-mediated secretion of human eosinophil granule-derived major basic protein
Melo, R.C.N., Spencer, L.A., Perez, S.A.C., Neves, J.S., Bafford, S.P., Morgan, E.S., Dvorak, A.M. and Weller, P.F.
*Lab. Invest., **89**, 769-781 (2009)*

Major basic protein (MBP), the predominant cationic protein of human eosinophil specific granules, is stored within crystalloid cores of these granules. Secretion of MBP contributes to the immunopathogenesis of varied diseases. Prior electron microscopy (EM) of eosinophils in sites of inflammation noted losses of granule cores in the absence of granule exocytosis and suggested that eosinophil granule proteins might be released through piecemeal degranulation (PMD), a secretory process mediated by transport vesicles. Because release of eosinophil granule-derived MBP through PMD has not been studied, we evaluated secretion of this cationic protein by human eosinophils. Intracellular localizations of MBP were studied within nonstimulated and eotaxin-stimulated human eosinophils by both immunofluorescence and a pre-embedding immunonanogold EM method that enables optimal epitope preservation and antigen access to membrane microdomains. In parallel, quantification of transport vesicles was assessed in eosinophils from a patient with hypereosinophilic syndrome (HES). Our data demonstrate vesicular trafficking of MBP within eotaxin-stimulated eosinophils. Vesicular compartments, previously implicated in transport from granules to the plasma membrane, including large vesiculotubular carriers termed eosinophil sombrero vesicles (EoSVs), were found to contain MBP. These secretory compartments were significantly increased in numbers within HES eosinophils. Moreover, in addition to granule-stored MBP, even unstimulated eosinophils contained appreciable amounts of MBP within secretory vesicles, as evidenced by immunonanogold EM and immunofluorescent colocalizations of MBP and CD63. These data suggest that eosinophil MBP, with its multiple extracellular activities, can be mobilized from granules by PMD into secretory vesicles and both granule- and secretory vesicle-stored pools of MBP are available for agonist-elicited secretion of MBP from human eosinophils. The recognition of PMD as a secretory process to release MBP is important to understand the pathological basis of allergic and other eosinophil-associated inflammatory diseases.

3.1290 Reversible Phosphorylation as a Molecular Switch to Regulate Plasma Membrane Targeting of Acylated SH4 Domain Proteins
Tournaviti, S., San Pietro, E., Terjung, S., Schafmeier, T., Wegehingel, S., Ritzerfeld, J., Schulz, J., Smith, D.F., Pepperkok, R. and Nickel, W.
*Traffic, **10(8)**, 1047-1060 (2009)*

Acylated SH4 domains represent N-terminal targeting signals that anchor peripheral membrane proteins such as Src kinases in the inner leaflet of plasma membranes. Here we provide evidence for a novel regulatory mechanism that may control the levels of SH4 proteins being associated with plasma membranes. Using a fusion protein of the SH4 domain of ***Leishmania*** HASPB and GFP as a model system, we demonstrate that threonine 6 is a substrate for phosphorylation. Substitution of threonine 6 by glutamate (to mimic a phosphothreonine residue) resulted in a dramatic redistribution from plasma membranes to intracellular sites with a particular accumulation in a perinuclear region. As shown by both

pharmacological inhibition and RNAi-mediated down-regulation of the threonine/ serine-specific phosphatases PP1 and PP2A, recycling back to the plasma membrane required dephosphorylation of threonine 6. We provide evidence that a cycle of phosphorylation and dephosphorylation may also be involved in intracellular targeting of other SH4 proteins such as the Src kinase Yes.

3.1291 Occludin oligomeric assemblies at tight junctions of the blood–brain barrier are altered by hypoxia and reoxygenation stress

McCaffrey, G., Willis, C.L., Staatz, W.D., Nametz, N., Quigley, C.A., Hom, S., Lochhead, J.J. and Davis, T.P.

J. Neurochem., ***110(1)***, *58-71 (2009)*

Hypoxic (low oxygen) and reperfusion (post-hypoxic reoxygenation) phases of stroke promote an increase in microvascular permeability at tight junctions (TJs) of the blood–brain barrier (BBB) that may lead to cerebral edema. To investigate the effect of hypoxia (Hx) and reoxygenation on oligomeric assemblies of the transmembrane TJ protein occludin, rats were subjected to either normoxia (Nx, 21% O_2, 60 min), Hx (6% O_2, 60 min), or hypoxia/reoxygenation (H/R, 6% O_2, 60 min followed by 21% O_2, 10 min). After treatment, cerebral microvessels were isolated, fractionated by detergent-free density gradient centrifugation, and occludin oligomeric assemblies associated with plasma membrane lipid rafts were solubilized by perfluoro-octanoic acid (PFO) exclusively as high molecular weight protein complexes. Analysis by non-reducing and reducing sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis/western blot of PFO-solubilized occludin revealed that occludin oligomeric assemblies co-localizing with 'TJ-associated' raft domains contained a high molecular weight 'structural core' that was resistant to disassembly by either SDS or a hydrophilic reducing agent *ex vivo*, and by Hx and H/R conditions *in vivo*. However, exposure of PFO-solubilized occludin oligomeric assemblies to SDS *ex vivo* revealed the non-covalent association of a significant amount of dimeric and monomeric occludin isoforms to the disulfide-bonded inner core, and dispersal of these non-covalently attached occludin subunits to lipid rafts of higher density *in vivo* was differentially promoted by Hx and H/R. Our data suggest a model of isoform interaction within occludin oligomeric assemblies at the BBB that enables occludin to simultaneously perform a structural role in inhibiting paracellular diffusion, and a signaling role involving interactions of dimeric and monomeric occludin isoforms with a variety of regulatory molecules within different plasma membrane lipid raft domains.

3.1292 Molecular and reverse genetic characterization of NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1) genes unravels their function in transcription and nucleotide excision repair in Arabidopsis thaliana

Liu, Z., Zhu, Y., Gao, J., Yu, F., Dong, A. and Shen, W-H.

Plant J., ***59(1)***, *27-38 (2009)*

Compared with the well-studied biochemical function of NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1) as a histone chaperone in nucleosome assembly/disassembly, the physiological roles of NAP1 remain largely uncharacterized. Here, we define the *NAP1* gene family members in Arabidopsis, examine their molecular properties, and use reverse genetics to characterize their biological roles. We show that the four AtNAP1-group proteins can form homodimers and heterodimers, can bind histone H2A, and are localized abundantly in the cytoplasm and weakly in the nucleus at steady state. AtNAP1;4 differs from the others by showing inhibitor-sensitive nucleocytoplasmic shuttling and tissue-specific expression, restricted to root segments and pollen grains. The other three *AtNAP1* genes are ubiquitously expressed in plants and the AtNAP1;3 protein is detected as the major isoform in seedlings. We show that disruption of the *AtNAP1*-group genes does not affect normal plant growth under our laboratory conditions. Interestingly, two allelic triple mutants, *Atnap1;1-1 Atnap1;2-1 Atnap1;3-1* and *Atnap1;1-1 Atnap1;2-1 Atnap1;3-2*, exhibit perturbed genome transcription, and show hypersensitivity to DNA damage caused by UV-C irradiation. We show that AtNAP1;3 binds chromatin, with enrichment at some genes involved in the nucleotide excision repair (NER) pathway, and that the expression of these genes is downregulated in the triple mutants. Taken together, our results highlight conserved and isoform-specific properties of AtNAP1 proteins, and unravel their function in the NER pathway of DNA damage repair.

3.1293 Repeated Cocaine Administration Decreases 5-HT_{2A} Receptor-Mediated Serotonergic Enhancement of Synaptic Activity in Rat Medial Prefrontal Cortex

Huang, C-C., Liang, Y-C., Lee, C-C., Wu, M-Y. and Hsu, K-S.

Neuropsychopharmacol., ***34***, *1979-1992 (2009)*

Neural adaptations in the medial prefrontal cortex (mPFC) are thought to be crucial in the development and maintenance of addictive behaviors. The mPFC receives a dense serotonergic (5-hydroxytryptamine, 5-HT) innervation from raphe nuclei and 5-HT exerts complex actions on mPFC pyramidal neurons. The present study, using a rat model of behavioral sensitization to cocaine, was designed to determine whether repeated cocaine exposure *in vivo* is capable of altering 5-HT-induced regulation of glutamatergic transmission in the mPFC. In layer V pyramidal neurons of the mPFC, application of 5-HT, through activation of 5-HT_{2A} receptors, induced a massive enhancement of spontaneous excitatory postsynaptic currents (sEPSCs). Repeated cocaine administration for 5 days resulted in an attenuation in the ability of 5-HT to enhance sEPSCs. This effect was prevented when cocaine was co-administered with the selective 5-HT_{2A} receptor antagonist ketanserin and was mimicked by repeated 5-HT_{2A} receptor agonist (-)4-iodo-2,5-dimethoxyphenylisopropylamine administration. Repeated cocaine administration is not associated with any changes in the levels of 5-HT_{2A} receptors or regulator of GTP-binding protein signaling 4. These results suggest that cocaine-induced inhibition of 5-HT_{2A} receptor-mediated enhancement of glutamatergic transmission in the mPFC may be caused, at least in part, by the impairment of coupling of 5-HT_{2A} receptors with GTP-binding proteins during cocaine withdrawal. These alterations in 5-HT_{2A} receptor responsiveness in the mPFC may be relevant to the development of behavioral sensitization and withdrawal effects following repeated cocaine administration.

3.1294 Caveolin-1-dependent and -independent membrane domains

Lay, S.L., Li, Q., Proschogo, N., Rodriguez, M., Gunaratnam, K., Cartland, S., Rentero, C., Jessup, W., Mitchell, T. and Gaus, K.

J. Lipid Res., ***50***, *1609-1620 (2009)*

Lipid rafts defined as cholesterol- and sphingomyelin-rich domains have been isolated from different cell types that vary greatly in their lipid profiles. Here, we investigated the contribution of the structural protein caveolin-1 (Cav1) to the overall lipid composition and domain abundance in mouse embryonic fibroblasts (MEFs) from wild-type (WT) or Cav1-deficient ($Cav1^{-/-}$) animals. Our findings show that Cav1 expression had no effect on free (membrane-associated) cholesterol levels. However, $Cav1^{-/-}$-deficient cells did have a higher proportion of sphingomyelin, decreased abundance of unsaturated phospholipids, and a trend toward shorter fatty acid chains in phosphatidylcholine. We isolated detergent-resistant membranes (DRMs), nondetergent raft domains (NDR), and cholesterol oxidase (CO)-sensitive domains and assessed the abundance of ordered domains in intact cells using the fluorescent dye Laurdan. Despite differences in phospholipid composition, we found that cholesterol levels in DRMs, NDR, and CO-sensitive domains were similar in both cell types. The data suggest that Cav1 is not required to target cholesterol to lipid rafts and that CO does not specifically oxidize caveolar cholesterol. In contrast, the abundance of ordered domains in adherent cells is reduced in $Cav1^{-/-}$ compared with WT MEFs, suggesting that cell architecture is critical in maintaining Cav1-induced lipid rafts.

3.1295 Human Dna2 Is a Nuclear and Mitochondrial DNA Maintenance Protein

Duxin, J.P., Dao, B., Martinsson, P., Rajala, N., Guittat, L., Campbell, J.L., Spelbrink, J.N. and Stewart, S.A.

Mol. Cell. Biol., ***29(15)***, *4274-4282 (2009)*

Dna2 is a highly conserved helicase/nuclease that in yeast participates in Okazaki fragment processing, DNA repair, and telomere maintenance. Here, we investigated the biological function of human Dna2 (hDna2). Immunofluorescence and biochemical fractionation studies demonstrated that hDna2 was present in both the nucleus and the mitochondria. Analysis of mitochondrial hDna2 revealed that it colocalized with a subfraction of DNA-containing mitochondrial nucleoids in unperturbed cells. Upon the expression of disease-associated mutant forms of the mitochondrial Twinkle helicase which induce DNA replication pausing/stalling, hDna2 accumulated within nucleoids. RNA interference-mediated depletion of hDna2 led to a modest decrease in mitochondrial DNA replication intermediates and inefficient repair of damaged mitochondrial DNA. Importantly, hDna2 depletion also resulted in the appearance of aneuploid cells and the formation of internuclear chromatin bridges, indicating that nuclear hDna2 plays a role in genomic DNA stability. Together, our data indicate that hDna2 is similar to its yeast counterpart and is a new addition to the growing list of proteins that participate in both nuclear and mitochondrial DNA maintenance.

3.1296 Endosomal Trafficking of HIV-1 Gag and Genomic RNAs Regulates Viral Egress

Molle, D., Segura-Morales, C., Camus, G., Berlioz-Torrent, C., Kjems, J., Basyuk, E. and Bertrand, E.

J. Biol. Chem., ***284(29)***, *19727-19743 (2009)*

HIV-1 Gag can assemble and generate virions at the plasma membrane, but it is also present in endosomes where its role remains incompletely characterized. Here, we show that HIV-1 RNAs and Gag are transported on endosomal vesicles positive for TiVamp, a v-SNARE involved in fusion events with the plasma membrane. Inhibition of endosomal traffic did not prevent viral release. However, inhibiting lysosomal degradation induced an accumulation of Gag in endosomes and increased viral production 7-fold, indicating that transport of Gag to lysosomes negatively regulates budding. This also suggested that endosomal Gag-RNA complexes could access retrograde pathways to the cell surface and indeed, depleting cells of TiVamp-reduced viral production. Moreover, inhibition of endosomal transport prevented the accumulation of Gag at sites of cellular contact. HIV-1 Gag could thus generate virions using two pathways, either directly from the plasma membrane or through an endosome-dependent route. Endosomal Gag-RNA complexes may be delivered at specific sites to facilitate cell-to-cell viral transmission.

3.1297 Heat shock protein 70 is secreted from endothelial cells by a non-classical pathway involving exosomes
Zhan, R., Leng, X., Liu, X., Wang, X., Gong, J., Yan, L., Wang, L., Wang, Y., Wang, X. and Qian, L-J.
Biochem. Biophys. Res. Comm., ***387****, 229-233 (2009)*

Emerging evidence suggests that a high level of circulating heat shock protein 70 (HSP70) correlates with a lower risk of vascular disease; however, the biological significance of this inverse relationship has not been explored. Herein, we report that oxidative low density lipoprotein (Ox-LDL) and homocysteine (Hcy) induce HSP70 release from endothelial cells. In rat endothelial cells, Ox-LDL and Hcy induced robust release of HSP70, independent of the classical route of endoplasmic reticulum/Golgi protein trafficking or the formation of lipid rafts. In contrast, Ox-LDL and Hcy significantly enhanced the exosomal secretory rate and increased the HSP70 content of exosomes. Exogenous HSP70 had no impact on LPS-, Ox-LDL- and Hcy-induced activation of endothelial cells, whereas HSP70 did activate monocytes alone, resulting in monocyte adhesion to endothelial cells. These results indicate that exosome-dependent secretion of HSP70 from endothelial cells provides a novel paracrine mechanism to regulate vascular endothelial functional integrity.

3.1298 In Vitro Intracellular Trafficking of Virulence Antigen during Infection by Yersinia pestis
DiMezzo, T.L., ruthel, G., Brueggemann, E.E., Hines, H.B., Ribot, W.J., Chapman, C.E., Powell, B.S. and Welkos, S.L.
PloSOne, ***4(7)****, e628 (2009)*

Yersinia pestis, the causative agent of plague, encodes several essential virulence factors on a 70 kb plasmid, including the *Yersinia* outer proteins (Yops) and a multifunctional virulence antigen (V). V is uniquely able to inhibit the host immune response; aid in the expression, secretion, and injection of the cytotoxic Yops via a type III secretion system (T3SS)-dependent mechanism; be secreted extracellularly; and enter the host cell by a T3SS-independent mechanism, where its activity is unknown. To elucidate the intracellular trafficking and target(s) of V, time-course experiments were performed with macrophages (MΦs) infected with *Y. pestis* or *Y. pseudotuberculosis* at intervals from 5 min to 6 h. The trafficking pattern was discerned from results of parallel microscopy, immunoblotting, and flow cytometry experiments. The MΦs were incubated with fluorescent or gold conjugated primary or secondary anti-V (antibodies [Abs]) in conjunction with organelle-associated Abs or dyes. The samples were observed for co-localization by immuno-fluorescence and electron microscopy. For fractionation studies, uninfected and infected MΦs were lysed and subjected to density gradient centrifugation coupled with immunoblotting with Abs to V or to organelles. Samples were also analyzed by flow cytometry after lysis and dual-staining with anti-V and anti-organelle Abs. Our findings indicate a co-localization of V with (1) endosomal proteins between 10–45 min of infection, (2) lysosomal protein(s) between 1–2 h of infection, (3) mitochondrial proteins between 2.5–3 h infection, and (4) Golgi protein(s) between 4–6 h of infection. Further studies are being performed to determine the specific intracellular interactions and role in pathogenesis of intracellularly localized V.

3.1299 Correction of the Disease Phenotype of Myocilin-Causing Glaucoma by a Natural Osmolyte
Jia, L-Y., gong, B., Pang, C-P., Huang, Y., Lam, D., S-C., Wang, N. And Yam, H-F.
Invest. Ophthalmol. Vis. Sci., ***50****, 3743-3749 (2009)*

PURPOSE. To characterize a novel Asp384Asn (D384N) mutant myocilin (MYOC) that causes juvenile-onset open-angle glaucoma (JOAG) and investigate the correction of mutant phenotype by a natural osmolyte, trimethylamine *N*-oxide (TMAO).
METHODS. A Chinese JOAG family was recruited and genomic DNA was extracted from peripheral blood obtained from 44 family members. Coding regions of the *MYOC* were sequenced. Two hundred individuals (>60 years old) without ocular hypertension or glaucoma were the control subjects. Full-length human wild-type *MYOC* cDNA was cloned in p3xFLAG-myc-CMV-25 and missense mutation was introduced by site-directed mutagenesis. Transfected human trabecular meshwork cells were treated with small-molecule chemical chaperones. Secreted MYOC was analyzed by combined immunoprecipitation-Western blot analysis. Intracellular myocilin was fractionated into Triton X-100-soluble and insoluble fractions, and analyzed by Western blot analysis. Intracellular aggregate and apoptosis were assayed by immunofluorescence. The effect of TMAO on subcellular myocilin distribution was analyzed by density gradient fractionation, followed by Western blot analysis.
RESULTS. A novel c.1150G>A change of *MYOC* was identified. Screening of optineurin, *WDR36*, and *CYP1B1* showed an absence of disease-causing polymorphisms. Mutated D384N myocilin had reduced solubility and was aggregation-prone and nonsecreted. Treatment of transfected cells with TMAO improved the solubility of the D384N mutant, which was corrected for secretion in a dose–response manner. TMAO reduced the distribution of the D384N mutant in the endoplasmic reticulum (ER), alleviated ER stress, and rescued cells from apoptosis.
CONCLUSIONS. The results indicate that TMAO, with chaperoning activity, facilitated the folding and secretion of mutant MYOC. This therapeutic approach assisted by a chemical chaperone can be developed for treating glaucoma.

3.1300 Harmonin in the Murine Retina and the Retinal Phenotypes of Ush1c-Mutant Mice and Human USH1C
Williams, D.S., Aleman, T.S., Lillo, C., Lopes, V.S., Hughes, L.C., Stone, E.M. and Jacobsen, S.G.
*Invest. Ophthalmol. Vis. Sci., **50**, 3881-3889 (2009)*

PURPOSE. To investigate the expression of harmonin in the mouse retina, test for ultrastructural and physiological mutant phenotypes in the retina of an *Ush1c* mutant mouse, and define in detail the retinal phenotype in human USH1C.
METHODS. Antibodies were generated against harmonin. Harmonin isoform distribution was examined by Western blot analysis and immunocytochemistry. Retinas of deaf circler (dfcr) mice, which possess mutant *Ush1c*, were analyzed by microscopy and electroretinography (ERG). Two siblings with homozygous 238_239insC (R80fs) *USH1C* mutations were studied with ERG, perimetry, and optical coherence tomography (OCT).
RESULTS. Harmonin isoforms a and c, but not b are expressed in the retina. Harmonin is concentrated in the photoreceptor synapse where the majority is postsynaptic. Dfcr mice do not undergo retinal degeneration and have normal synaptic ultrastructure and ERGs. USH1C patients had abnormal rod and cone ERGs. Rod- and cone-mediated sensitivities and retinal laminar architecture were normal across 50°–60° of visual field. A transition zone to severely abnormal function and structure was present at greater eccentricities.
CONCLUSIONS. The largest harmonin isoforms are not expressed in the retina. A major retinal concentration of harmonin is in the photoreceptor synapses, both pre- and post-synaptically. The dfcr mouse retina is unaffected by its mutant *Ush1c*. Patients with USH1C retained regions of normal central retina surrounded by degeneration. Perhaps the human disease is simply more aggressive than that in the mouse. Alternatively, the dfcr mouse may be a model for nonsyndromic deafness, due to the nonpathologic effect of its mutation on the retinal isoforms.

3.1301 Slit Diaphragms Contain Tight Junction Proteins
Fukasawa, H., Borheimer, S., Kudlicka, K. And Farquhar, M.G.
*J. Am. Soc. Nephrol., **20**, 1491-1503 (2009)*

Slit diaphragms are essential components of the glomerular filtration apparatus, as changes in these junctions are the hallmark of proteinuric diseases. Slit diaphragms, considered specialized adherens junctions, contain both unique membrane proteins (*e.g.*, nephrin, podocin, and Neph1) and typical adherens junction proteins (*e.g.*, P-cadherin, FAT, and catenins). Whether slit diaphragms also contain tight junction proteins is unknown. Here, immunofluorescence, immunogold labeling, and cell fractionation demonstrated that rat slit diaphragms contain the tight junction proteins JAM-A (junctional adhesion molecule A), occludin, and cingulin. We found these proteins in the same protein complexes as nephrin, podocin, CD2AP, ZO-1, and Neph1 by cosedimentation, coimmunoprecipitation, and pull-down assays.

PAN nephrosis increased the protein levels of JAM-A, occludin, cingulin, and ZO-1 several-fold in glomeruli and loosened their attachment to the actin cytoskeleton. These data extend current information about the molecular composition of slit diaphragms by demonstrating the presence of tight junction proteins, although slit diaphragms lack the characteristic morphologic features of tight junctions. The contribution of these proteins to the assembly of slit diaphragms and potential signaling cascades requires further investigation.

3.1302 TNF-α-induced up-regulation of pro-inflammatory cytokines is reduced by phosphatidylcholine in intestinal epithelial cells

Treede, I., Braun, A., Jeliaskova, P., Giese, T., Füllekrug, J., Griffiths, G., Stremmel, W. and Ehehalt, R.
BMC Gastroenterol., ***9***, *53-63 (2009)*

Background
Phosphatidylcholine (PC) is a major lipid of the gastrointestinal mucus layer. We recently showed that mucus from patients suffering from ulcerative colitis has low levels of PC. Clinical studies reveal that the therapeutic addition of PC to the colonic mucus using slow release preparations is beneficial. The positive role of PC in this disease is still unclear; however, we have recently shown that PC has an intrinsic anti-inflammatory property. It could be demonstrated that the exogenous application of PC inhibits membrane-dependent actin assembly and TNF-α-induced nuclear NF-κB activation. We investigate here in more detail the hypothesis that the exogenous application of PC has anti-inflammatory properties.
Methods
PC species with different fatty acid side chains were applied to differentiated and non-differentiated Caco-2 cells treated with TNF-α to induce a pro-inflammatory response. We analysed TNF-α-induced NF-κB-activation via the transient expression of a NF-κB-luciferase reporter system. Pro-inflammatory gene transcription was detected with the help of a quantitative real time (RT)-PCR analysis. We assessed the binding of TNF-α to its receptor by FACS and analysed lipid rafts by isolating detergent resistant membranes (DRMs).
Results
The exogenous addition of all PC species tested significantly inhibited TNF-α-induced pro-inflammatory signalling. The expression levels of IL-8, ICAM-1, IP-10, MCP-1, TNF-α and MMP-1 were significantly reduced after PC pre-treatment for at least two hours. The effect was comparable to the inhibition of NF-kB by the NF-kB inhibitor SN 50 and was not due to a reduced binding of TNF-α to its receptor or a decreased surface expression of TNF-α receptors. PC was also effective when applied to the apical side of polarised Caco-2 cultures if cells were stimulated from the basolateral side. PC treatment changed the compartmentation of the TNF-α-receptors 1 and 2 to DRMs.
Conclusion
PC induces a prolonged inhibition of TNF-α-induced pro-inflammatory signalling. This inhibition may be caused by a shift of the TNF-α receptors at the surface to lipid rafts. Our results may offer a potential molecular explanation for the positive role of PC seen in clinical studies for the treatment of ulcerative colitis.

3.1303 Raptor Binds the SAIN (Shc and IRS-1 NPXY Binding) Domain of Insulin Receptor Substrate-1 (IRS-1) and Regulates the Phosphorylation of IRS-1 at Ser-636/639 by mTOR

Tzatsos, A.
J. Biol. Chem., ***284(34)***, *22525-22534 (2009)*

In normal physiological states mTOR phosphorylates and activates Akt. However, under diabetic-mimicking conditions mTOR inhibits phosphatidylinositol (PI) 3-kinase/Akt signaling by phosphorylating insulin receptor substrate-1 (IRS-1) at Ser-636/639. The molecular basis for the differential effect of mTOR signaling on Akt is poorly understood. Here, it has been shown that knockdown of mTOR, Raptor, and mLST8, but not Rictor and mSin1, suppresses insulin-stimulated phosphorylation of IRS-1 at Ser-636/639 and stabilizes IRS-1 after long term insulin stimulation. This phosphorylation depends on the PI 3-kinase/PDK1 axis but is Akt-independent. At the molecular level, Raptor binds the SAIN (Shc and IRS-1 NP*X*Y binding) domain of IRS-1 and regulates the phosphorylation of IRS-1 at Ser-636/639 by mTOR. IRS-1 lacking the SAIN domain does not interact with Raptor, is not phosphorylated at Ser-636/639, and favorably interacts with PI 3-kinase. Overall, these data provide new insights in the molecular mechanisms by which mTORC1 inhibits PI 3-kinase/Akt signaling at the level of IRS-1 and suggest that mTOR signaling toward Akt is scaffold-dependent.

3.1304 Hypoxia-inducible Factor Prolyl-4-hydroxylase PHD2 Protein Abundance Depends on Integral Membrane Anchoring of FKBP38

Barth, S., Edlich, F., Berchner-Pfannschmidt, U., Gneuss, S., Jahreis, G., Hasgall, P.A., Fandrey, J., Wenger, R.H. and Camenisch, G.
J. Biol. Chem., ***284(34)***, *23046-23058 (2009)*

Prolyl-4-hydroxylase domain (PHD) proteins are 2-oxoglutarate and dioxygen-dependent enzymes that mediate the rapid destruction of hypoxia-inducible factor αsubunits. Whereas PHD1 and PHD3 proteolysis has been shown to be regulated by Siah2 ubiquitin E3 ligase-mediated polyubiquitylation and proteasomal destruction, protein regulation of the main oxygen sensor responsible for hypoxia-inducible factor α regulation, PHD2, remained unknown. We recently reported that the FK506-binding protein (FKBP) 38 specifically interacts with PHD2 and determines PHD2 protein stability in a peptidyl-prolyl *cis-trans* isomerase-independent manner. Using peptide array binding assays, fluorescence spectroscopy, and fluorescence resonance energy transfer analysis, we defined a minimal linear glutamate-rich PHD2 binding domain in the N-terminal part of FKBP38 and showed that this domain forms a high affinity complex with PHD2. Vice versa, PHD2 interacted with a non-linear N-terminal motif containing the MYND (myeloid, Nervy, and DEAF-1)-type Zn^{2+} finger domain with FKBP38. Biochemical fractionation and immunofluorescence analysis demonstrated that PHD2 subcellular localization overlapped with FKBP38 in the endoplasmic reticulum and mitochondria. An additional fraction of PHD2 was found in the cytoplasm. *In cellulo* PHD2/FKBP38 association, as well as regulation of PHD2 protein abundance by FKBP38, is dependent on membrane- anchored FKBP38 localization mediated by the C-terminal transmembrane domain. Mechanistically our data indicate that PHD2 protein stability is regulated by a ubiquitin-independent proteasomal pathway involving FKBP38 as adaptor protein that mediates proteasomal interaction. We hypothesize that FKBP38-bound PHD2 is constantly degraded whereas cytosolic PHD2 is stable and able to function as an active prolyl-4-hydroxylase.

3.1305 Enrichment and analysis of secretory lysosomes from lymphocyte populations

Schmidt, H., Gelhaus, C., Lucius, R., Nebendahl, M., Leippe, M. and Janssen, O.
BMC Immunol., ***10***, *41-52 (2009)*

Background
In specialized cells, such as mast cells, macrophages, T lymphocytes and Natural Killer cells in the immune system and for instance melanocytes in the skin, secretory lysosomes (SL) have evolved as bifunctional organelles that combine degradative and secretory properties. Mutations in lysosomal storage, transport or sorting molecules are associated with severe immunodeficiencies, autoimmunity and (partial) albinism. In order to analyze the function and content of secretory lysosomes in different cell populations, an efficient enrichment of these organelles is mandatory.
Results
Based on a combination of differential and density gradient centrifugation steps, we provide a protocol to enrich intact SL from expanded hematopoietic cells, here T lymphocytes and Natural Killer cells. Individual fractions were initially characterized by Western blotting using antibodies against an array of marker proteins for intracellular compartments. As indicated by the presence of LAMP-3 (CD63) and FasL (CD178), we obtained a selective enrichment of SL in one of the resulting organelle fractions. The robustness and reproducibility of the applied separation protocol was examined by a high-resolution proteome analysis of individual SL preparations of different donors by 2D difference gel electrophoresis (2D-DIGE).
Conclusion
The provided protocol is readily applicable to enrich and isolate intact secretory vesicles from individual cell populations. It can be used to compare SL of normal and transformed cell lines or primary cell populations from healthy donors and patients with lysosomal storage or transport diseases, or from corresponding mutant mice. A subsequent proteome analysis allows the characterization of molecules involved in lysosomal maturation and cytotoxic effector function at high-resolution.

3.1306 Ebolavirus Glycoprotein GP Masks both Its Own Epitopes and the Presence of Cellular Surface Proteins

Reynard, O., Borowiak, M., Volchkova, V.A., Delpeut, S., Mateo, M. and Volchkov, V.E.
J.Virol., ***83(18)***, *9596-9601 (2009)*

Ebolavirus (EBOV) is the etiological agent of a severe hemorrhagic fever with a high mortality rate. The spike glycoprotein (GP) is believed to be one of the major determinants of virus pathogenicity. In this

study, we demonstrated the molecular mechanism responsible for the downregulation of surface markers caused by EBOV GP expression. We showed that expression of mature GP on the plasma membrane results in the masking of cellular surface proteins, including major histocompatibility complex class I. Overexpression of GP also results in the masking of certain antigenic epitopes on GP itself, causing an illusory effect of disappearance from the plasma membrane.

3.1307 Retinal Pigment Epithelium Defects in Humans and Mice with Mutations in MYO7A: Imaging Melanosome-Specific Autofluorescence
Gibbs, D., Cideciyan, A.V., Jacobson, S.G. and Williams, D.S.
Invest. Ophthalmol. Vis. Sci., ***50,*** *4386-4393 (2009)*

PURPOSE. Usher syndrome (USH) is a genetically heterogeneous disease with autosomal recessive deafness and blindness. Gene therapy is under development for use in the most common genetic variant of USH1, USH1B, which is caused by mutations in the *MYO7A* gene. This study was undertaken to identify an imaging method for noninvasively monitoring the RPE component of the USH1B disease.
METHODS. NIR-autofluorescence (NIR-AF) was examined in USH1B patients with scanning laser ophthalmoscopy, and retinal thickness with spectral-domain optical coherence tomography. *Myo7a*-null mouse retinas and purified RPE melanosomes were analyzed by spectral deconvolution confocal microscopy.
RESULTS. In USH1B patients, NIR-AF was normal in regions of retained photoreceptors and abnormal in regions lacking photoreceptors. Subtle changes in NIR-AF were associated with intermediate photoreceptor loss. In ex vivo mouse retinas, the NIR-AF source was traced to the melanosomes in the RPE and choroid. Purified RPE melanosomes emitted the same signal. Fluorophores, excited by long-wavelength light, were evident throughout the apical RPE of WT mouse eyecups. In *Myo7a*-null eyecups, these fluorophores had a more restricted distribution. They were absent from the apical processes of the RPE, thus correlating with the melanosome localization defects described previously by conventional microscopy.
CONCLUSIONS. The data indicate that melanosomes in the RPE and choroid are the dominant source of NIR-AF from the posterior region of the eye. NIR-AF is a novel tool that provides sensitive and label-free imaging of the retina and RPE and is currently the only melanosome-specific, noninvasive technique for monitoring RPE disease in new therapeutic initiatives for retinal degenerations.

3.1308 Mapping Organelle Proteins and Protein Complexes in Drosophila melanogaster
Tan, D.J.L., Dvinge, H., Christoforou, A., Bertone, P., Martinez Arias, A. and Lilley, K.S.
J. Proteeome Res., ***8****, 2667-2678 (2009)*

Many proteins within eukaryotic cells are organized spatially and functionally into membrane bound organelles and complexes. A protein's location thus provides information about its function. Here, we apply LOPIT, a mass-spectrometry based technique that simultaneously maps proteins to specific subcellular compartments, to *Drosophila* embryos. We determine the subcellular distribution of hundreds of proteins, and protein complexes. Our results reveal the potential of LOPIT to provide average snapshots of cells.

3.1309 Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity
Gibbins, D.J., Ciaudo, C., Erhardt, M. and Voinnet, O.
Nature Cell Biol., ***11(9)****, 1143-1149 (2009)*

In animals, P-bodies or GW-bodies appear to cause the congregation of proteins involved in microRNA (miRNA)-mediated post-transcriptional silencing. The localization of P-bodies does not overlap with that of known organelles and are thus considered independent of lipid bilayers. Nonetheless, an miRNA effector protein, argonaute 2 (AGO2), was initially identified as membrane-associated, and some miRNAs have been found in secreted vesicles (exosomes) that derive from endo-lysosomal compartments called multivesicular bodies (MVBs). Proteins can be sorted in a ubiquitin-dependent manner into MVBs by three heteromeric subcomplexes, collectively termed ESCRT (endosomal sorting complex required for transport), to be further secreted in exosomes and/or degraded by the lysosome. Here we show that GW-bodies containing GW182 and AGO2, two main components of the RNA-induced silencing complex (RISC), are distinct from P-bodies due to their congregation with endosomes and MVBs. Moreover, miRNAs and miRNA-repressible mRNAs are enriched at these cellular membranes, suggesting that

endosomes and/or MVBs are sites of miRNA-loaded RISC (miRISC) accumulation and, possibly, action. We further show that purified exosome-like vesicles secreted by MVBs are considerably enriched in GW182, but not P-body components, AGO2 or miRNA-repressible mRNA. Moreover, cells depleted of some ESCRT components show compromised miRNA-mediated gene silencing and over-accumulate GW182, which associates with ubiquitylated proteins. Therefore, GW182, possibly in association with a fraction of miRNA-loaded AGO2, is sorted into MVBs for secretion and/or lysosomal degradation. We propose that this process promotes continuous assembly or disassembly of membrane-associated miRISCs, which is possibly required for miRNA loading or target recognition and subsequent silencing.

3.1310 Silencing by small RNAs is linked to endosomal trafficking
Lee, Y.S., Pressman, S., Andress, A.P., Kim, K., White, J.L., Cassidy, J.J., Li, X., Lubell, K., Lim, D.H., Cho, I.S., Nakahara, K., Preall, J.B., Bellare, P., Sontheimer, E.J. and Carthew, R.W.
Nature Cell Biol., ***11(9)***, *1150-1156 (2009)*

Small RNAs direct RNA-induced silencing complexes (RISCs) to regulate stability and translation of mRNAs[1, 2]. RISCs associated with target mRNAs often accumulate in discrete cytoplasmic foci known as GW-bodies[3]. However, RISC proteins can associate with membrane compartments such as the Golgi and endoplasmic reticulum[4]. Here, we show that GW-bodies are associated with late endosomes (multivesicular bodies, MVBs). Blocking the maturation of MVBs into lysosomes by loss of the tethering factor HPS4 (ref. 5) enhances short interfering RNA (siRNA)- and micro RNA (miRNA)-mediated silencing in *Drosophila melanogaster* and humans. It also triggers over-accumulation of GW-bodies. Blocking MVB formation by ESCRT (endosomal sorting complex required for transport)[6] depletion results in impaired miRNA silencing and loss of GW-bodies. These results indicate that active RISCs are physically and functionally coupled to MVBs. We further show that MVBs promote the competence of RISCs in loading small RNAs. We suggest that the recycling of RISCs is promoted by MVBs, resulting in RISCs more effectively engaging with small RNA effectors and possibly target RNAs. It may provide a means to enhance the dynamics of RNA silencing in the cytoplasm.

3.1311 Rab35 Controls Actin Bundling by Recruiting Fascin as an Effector Protein
Zhang, J., Fonovic, M., Suyama, K., Bogyo, M. and Scott, M.P.
Science, ***325***, *1250-1254 (2009)*

Actin filaments are key components of the eukaryotic cytoskeleton that provide mechanical structure and generate forces during cell shape changes, growth, and migration. Actin filaments are dynamically assembled into higher-order structures at specified locations to regulate diverse functions. The Rab family of small guanosine triphosphatases is evolutionarily conserved and mediates intracellular vesicle trafficking. We found that Rab35 regulates the assembly of actin filaments during bristle development in Drosophila and filopodia formation in cultured cells. These effects were mediated by the actin-bundling protein fascin, which directly associated with active Rab35. Targeting Rab35 to the outer mitochondrial membrane triggered actin recruitment, demonstrating a role for an intracellular trafficking protein in localized actin assembly.

3.1312 Reduced Amyloid Deposition in Mice Overexpressing RTN3 Is Adversely Affected by Preformed Dystrophic Neurites
Shi, Q., Prior, M., He, W., tang, X., Hu, X. and Yan, R.
J. Neurosci., ***29(29),*** *9163-9173 (2009)*

Reticulon 3 (RTN3) was initially identified as a negative modulator of BACE1, an enzyme that cleaves amyloid precursor protein (APP) to release β-amyloid peptide. Interestingly, RTN3 can also form aggregates after accumulation, and increased RTN3 aggregation correlates with the formation of RTN3 immunoreactive dystrophic neurites (RIDNs) in brains of Alzheimer's cases. Transgenic mice expressing RTN3 alone develop RIDNs in their hippocampus but not in their cortex. To determine the *in vivo* effects of RTN3 and preformed RIDNs on amyloid deposition, we crossed bitransgenic mice expressing APP and presenilin 1 (PS1) mutations with mice overexpressing RTN3. We found that amyloid deposition in cortex, the hippocampal CA3 region, and dentate gyrus was significantly reduced in triple transgenic mice compared with bitransgenic controls. However, reduction of amyloid deposition in the hippocampal CA1 region, where RIDNs predominantly formed before amyloid deposition, was less significant. Hence, preformed RTN3 aggregates in RIDNs clearly offset the negative modulation of BACE1 activity by RTN3. Furthermore, our study indicates that the increased expression of RTN3 could result in an alteration of BACE1 intracellular trafficking by retaining more BACE1 in the endoplasmic reticulum compartment

where cleavage of APP by BACE1 is less favored. Our results suggest that inhibition of RTN3 aggregation is likely to be beneficial by reducing both amyloid deposition and the formation RIDNs.

3.1313 Correction of the Disease Phenotype of Myocilin-Causing Glaucoma by a Natural Osmolyte
Jia, L-Y., Gong, B., Pang, C-P., Huang, Y., Lam, D.S-C., Wang, N. and Yam, G.H-F.
Invest. Ophthalmol. Vis. Sci., ***50(8)****, 3743-3749 (2009)*

PURPOSE. To characterize a novel Asp384Asn (D384N) mutant myocilin (MYOC) that causes juvenile-onset open-angle glaucoma (JOAG) and investigate the correction of mutant phenotype by a natural osmolyte, trimethylamine *N*-oxide (TMAO).
METHODS. A Chinese JOAG family was recruited and genomic DNA was extracted from peripheral blood obtained from 44 family members. Coding regions of the *MYOC* were sequenced. Two hundred individuals (>60 years old) without ocular hypertension or glaucoma were the control subjects. Full-length human wild-type *MYOC* cDNA was cloned in p3xFLAG-myc-CMV-25 and missense mutation was introduced by site-directed mutagenesis. Transfected human trabecular meshwork cells were treated with small-molecule chemical chaperones. Secreted MYOC was analyzed by combined immunoprecipitation-Western blot analysis. Intracellular myocilin was fractionated into Triton X-100-soluble and insoluble fractions, and analyzed by Western blot analysis. Intracellular aggregate and apoptosis were assayed by immunofluorescence. The effect of TMAO on subcellular myocilin distribution was analyzed by density gradient fractionation, followed by Western blot analysis.
RESULTS. A novel c.1150G>A change of *MYOC* was identified. Screening of optineurin, *WDR36*, and *CYP1B1* showed an absence of disease-causing polymorphisms. Mutated D384N myocilin had reduced solubility and was aggregation-prone and nonsecreted. Treatment of transfected cells with TMAO improved the solubility of the D384N mutant, which was corrected for secretion in a dose–response manner. TMAO reduced the distribution of the D384N mutant in the endoplasmic reticulum (ER), alleviated ER stress, and rescued cells from apoptosis.
CONCLUSIONS. The results indicate that TMAO, with chaperoning activity, facilitated the folding and secretion of mutant MYOC. This therapeutic approach assisted by a chemical chaperone can be developed for treating glaucoma.

3.1314 Harmonin in the Murine Retina and the Retinal Phenotypes of Ush1c-Mutant Mice and Human USH1C
Williams, D.S., Aleman, T.S., Lillo, C., Lopes, V.S., Hughes, L.C., Stone, E.M. and Jacobson, S.G.
Invest. Ophthalmol. Vis. Sci., ***50(8)****, 3881-3889 (2009)*

PURPOSE. To investigate the expression of harmonin in the mouse retina, test for ultrastructural and physiological mutant phenotypes in the retina of an *Ush1c* mutant mouse, and define in detail the retinal phenotype in human USH1C.
METHODS. Antibodies were generated against harmonin. Harmonin isoform distribution was examined by Western blot analysis and immunocytochemistry. Retinas of deaf circler (dfcr) mice, which possess mutant *Ush1c*, were analyzed by microscopy and electroretinography (ERG). Two siblings with homozygous 238_239insC (R80fs) *USH1C* mutations were studied with ERG, perimetry, and optical coherence tomography (OCT).
RESULTS. Harmonin isoforms a and c, but not b are expressed in the retina. Harmonin is concentrated in the photoreceptor synapse where the majority is postsynaptic. Dfcr mice do not undergo retinal degeneration and have normal synaptic ultrastructure and ERGs. USH1C patients had abnormal rod and cone ERGs. Rod- and cone-mediated sensitivities and retinal laminar architecture were normal across 50°–60° of visual field. A transition zone to severely abnormal function and structure was present at greater eccentricities.
CONCLUSIONS. The largest harmonin isoforms are not expressed in the retina. A major retinal concentration of harmonin is in the photoreceptor synapses, both pre- and post-synaptically. The dfcr mouse retina is unaffected by its mutant *Ush1c.* Patients with USH1C retained regions of normal central retina surrounded by degeneration. Perhaps the human disease is simply more aggressive than that in the mouse. Alternatively, the dfcr mouse may be a model for nonsyndromic deafness, due to the nonpathologic effect of its mutation on the retinal isoforms.

3.1315 Nogo-B Receptor Stabilizes Niemann-Pick Type C2 Protein and Regulates Intracellular Cholesterol Trafficking
Harrison, K.D., Miao, R.Q., Fernandez-Hernando, C.F., Suarez, Y., Davalos, A. and Sessa, W.C.
Cell Metabolism, ***10****, 208-218 (2009)*

The Nogo-B receptor (NgBR) is a recently identified receptor for the N terminus of reticulon 4B/Nogo-B. Other than its role in binding Nogo-B, little is known about the biology of NgBR. To elucidate a basic cellular role for NgBR, we performed a yeast two-hybrid screen for interacting proteins, using the C-terminal domain as bait, and identified Niemann-Pick type C2 protein (NPC2) as an NgBR-interacting protein. NPC2 protein levels are increased in the presence of NgBR, and NgBR enhances NPC2 protein stability. NgBR localizes primarily to the endoplasmic reticulum (ER) and regulates the stability of nascent NPC2. RNAi-mediated disruption of NgBR or genetic deficiency in NgBR lead to a decrease in NPC2 levels, increased intracellular cholesterol accumulation, and a loss of sterol sensing, all hallmarks of an NPC2 mutation. These data identify NgBR as an NPC2-interacting protein and provide evidence of a role for NgBR in intracellular cholesterol trafficking.

3.1316 Contributions of quantitative proteomics to understanding membrane microdomains
Zheng, Y. and Foster, L.J.
*J. Lipid Res., **50**, 1976-1985 (2009)*

Membrane microdomains, e.g., lipid rafts and caveolae, are crucial cell surface organelles responsible for many cellular signaling and communication events, which makes the characterization of their proteomes both interesting and valuable. They are large cellular complexes comprised of specific proteins and lipids, yet they are simple enough in composition to be amenable to modern LC/MS/MS methods for proteomics. However, the proteomic characterization of membrane microdomains by traditional qualitative mass spectrometry is insufficient for distinguishing true components of the microdomains from copurifying contaminants or for evaluating dynamic changes in the proteome compositions. In this review, we discuss the contributions quantitative proteomics has made to our understanding of the biology of membrane microdomains.

3.1317 Assembly of Arenavirus Envelope Glycoprotein GPC in Detergent-Soluble Membrane Microdomains
Agnihothram, S.S., Dancho, B., Grant, K.W., Grimes, M.L., Lyles, D.S. and Nunberg, J.H.
*J. Virol., **83(19)**, 9890-9900 (2009)*

The family Arenaviridae includes a number of highly pathogenic viruses that are responsible for acute hemorrhagic fevers in humans. Genetic diversity among arenavirus species in their respective rodent hosts supports the continued emergence of new pathogens. In the absence of available vaccines or therapeutic agents, the hemorrhagic fever arenaviruses remain a serious public health and biodefense concern. Arenaviruses are enveloped virions that assemble and bud from the plasma membrane. In this study, we have characterized the microdomain organization of the virus envelope glycoprotein (GPC) on the cell surface by using immunogold electron microscopy. We find that Junín virus (JUNV) GPC clusters into discrete microdomains of 120 to 160 nm in diameter and that this property of GPC is independent of its myristoylation and of coexpression with the virus matrix protein Z. In cells infected with the Candid#1 strain of JUNV, and in purified Candid#1 virions, these GPC microdomains are soluble in cold Triton X-100 detergent and are thus distinct from conventional lipid rafts, which are utilized by numerous other viruses for assembly. Virion morphogenesis ultimately requires colocalization of viral components, yet our dual-label immunogold staining studies failed to reveal a spatial association of Z with GPC microdomains. This observation may reflect either rapid Z-dependent budding of virus-like particles upon coassociation or a requirement for additional viral components in the assembly process. Together, these results provide new insight into the molecular basis for arenavirus morphogenesis.

3.1318 Characterization of Hepatitis C Virus Core Protein Multimerization and Membrane Envelopment: Revelation of a Cascade of Core-Membrane Interactions
Ai, L-S., Lee, Y-W. and Chen, S.S.-L.
*J. Virol., **83(19)**, 9923-9939 (2009)*

The molecular basis underlying hepatitis C virus (HCV) core protein maturation and morphogenesis remains elusive. We characterized the concerted events associated with core protein multimerization and interaction with membranes. Analyses of core proteins expressed from a subgenomic system showed that the signal sequence located between the core and envelope glycoprotein E1 is critical for core association with endoplasmic reticula (ER)/late endosomes and the core's envelopment by membranes, which was judged by the core's acquisition of resistance to proteinase K digestion. Despite exerting an inhibitory

effect on the core's association with membranes, (Z-LL)2-ketone, a specific inhibitor of signal peptide peptidase (SPP), did not affect core multimeric complex formation, suggesting that oligomeric core complex formation proceeds prior to or upon core attachment to membranes. Protease-resistant core complexes that contained both innate and processed proteins were detected in the presence of (Z-LL)2-ketone, implying that core envelopment occurs after intramembrane cleavage. Mutations of the core that prevent signal peptide cleavage or coexpression with an SPP loss-of-function D219A mutant decreased the core's envelopment, demonstrating that SPP-mediated cleavage is required for core envelopment. Analyses of core mutants with a deletion in domain I revealed that this domain contains sequences crucial for core envelopment. The core proteins expressed by infectious JFH1 and Jc1 RNAs in Huh7 cells also assembled into a multimeric complex, associated with ER/late-endosomal membranes, and were enveloped by membranes. Treatment with (Z-LL)2-ketone or coexpression with D219A mutant SPP interfered with both core envelopment and infectious HCV production, indicating a critical role of core envelopment in HCV morphogenesis. The results provide mechanistic insights into the sequential and coordinated processes during the association of the HCV core protein with membranes in the early phase of virus maturation and morphogenesis.

3.1319 Printor, a Novel TorsinA-interacting Protein Implicated in Dystonia Pathogenesis
Giles, L.M., Li, L. and Chin, L-S.
J. Biol. Chem., ***284(32)***, *21765-21775 (2009)*

Early onset generalized dystonia (DYT1) is an autosomal dominant neurological disorder caused by deletion of a single glutamate residue (torsinA ΔE) in the C-terminal region of the AAA^+ (ATPases associated with a variety of cellular activities) protein torsinA. The pathogenic mechanism by which torsinA ΔE mutation leads to dystonia remains unknown. Here we report the identification and characterization of a 628-amino acid novel protein, printor, that interacts with torsinA. Printor co-distributes with torsinA in multiple brain regions and co-localizes with torsinA in the endoplasmic reticulum. Interestingly, printor selectively binds to the ATP-free form but not to the ATP-bound form of torsinA, supporting a role for printor as a cofactor rather than a substrate of torsinA. The interaction of printor with torsinA is completely abolished by the dystonia-associated torsinA ΔE mutation. Our findings suggest that printor is a new component of the DYT1 pathogenic pathway and provide a potential molecular target for therapeutic intervention in dystonia.

3.1320 Functionally Relevant Domains of the Prion Protein Identified *In Vivo*
Baumann, F., Pahnke, J., Radovanovic, I., Rülicke, T., Bremer, J., Tolnay, M. And Aguzzi, A.
PloSOne, ***4(9)***, *e607 (2009)*

The prion consists essentially of PrP^{Sc}, a misfolded and aggregated conformer of the cellular protein PrP^{C}. Whereas PrP^{C} deficient mice are clinically healthy, expression of PrP^{C} variants lacking its central domain ($PrP_{\Delta CD}$), or of the PrP-related protein Dpl, induces lethal neurodegenerative syndromes which are repressed by full-length PrP. Here we tested the structural basis of these syndromes by grafting the amino terminus of PrP^{C} (residues 1–134), or its central domain (residues 90–134), onto Dpl. Further, we constructed a soluble variant of the neurotoxic $PrP_{\Delta CD}$ mutant that lacks its glycosyl phosphatidyl inositol (GPI) membrane anchor. Each of these modifications abrogated the pathogenicity of Dpl and $PrP_{\Delta CD}$ in transgenic mice. The PrP-Dpl chimeric molecules, but not anchorless $PrP_{\Delta CD}$, ameliorated the disease of mice expressing truncated PrP variants. We conclude that the amino proximal domain of PrP exerts a neurotrophic effect even when grafted onto a distantly related protein, and that GPI-linked membrane anchoring is necessary for both beneficial and deleterious effects of PrP and its variants.

3.1321 Lipid rafts serve as signaling platforms for Tie2 receptor tyrosine kinase in vascular endothelial cells
Katoh, S-Y., Kamimoto, T., Yamakawa, D. and Takakura, N.
Exp. Cell Res., ***315***, *2818-2823 (2009)*

The Tie2 receptor tyrosine kinase plays a pivotal role in vascular and hematopoietic development. The major intracellular signaling systems activated by Tie2 in response to Angiopoietin-1 (Ang1) include the Akt and Erk1/2 pathways. Here, we investigated the role of cholesterol-rich plasma membrane microdomains (lipid rafts) in Tie2 regulation. Tie2 could not be detected in the lipid raft fraction of human umbilical vein endothelial cells (HUVECs) unless they were first stimulated with Ang1. After stimulation, a minor fraction of Tie2 associated tightly with the lipid rafts. Treatment of HUVECs with the lipid raft disrupting agent methyl-β-cyclodextrin selectively inhibited Ang1-induced Akt phosphorylation, but not Erk1/2 phosphorylation. It has been reported that inhibition of FoxO activity is an important mechanism

for Ang1-stimulated Tie2-mediated endothelial function. Consistent with this, we found that phosphorylation of FoxO mediated by Tie2 activation was attenuated by lipid raft disruption. Therefore, we propose that lipid rafts serve as signaling platforms for Tie2 receptor tyrosine kinase in vascular endothelial cells, especially for the Akt pathway.

3.1322 Lipid dependence of ABC transporter localization and function
Klappe, K., Hummel, I., Hoekstra, D. And Kok, J.W.
*Chemistry and Physics of Lipids, **161**, 57-64 (2009)*

Lipid rafts have been implicated in many cellular functions, including protein and lipid transport and signal transduction. ATP-binding cassette (ABC) transporters have also been localized in these membrane domains. In this review the evidence for this specific localization will be evaluated and discussed in terms of relevance to ABC transporter function. We will focus on three ABC transporters of the A, B and C subfamily, respectively. Two of these transporters are relevant to multidrug resistance in tumor cells (Pgp/ABCB1 and MRP1/ABCC1), while the third (ABCA1) is extensively studied in relation to the reverse cholesterol pathway and cellular cholesterol homeostasis. We will attempt to derive a generalized model of lipid rafts to which they associate based on the use of various different lipid raft isolation procedures. In the context of lipid rafts, modulation of ABC transporter localization and function by two relevant lipid classes, i.e. sphingolipids and cholesterol, will be discussed.

3.1323 Mitochondrial Expression and Functional Activity of Breast Cancer Resistance Protein in Different Multiple Drug-Resistant Cell Lines
Solazzo, M., Fantappie, O., D'Amico, M., Sassoli, C., Tani, A., Cipriani, G., Bogani, C., Formigli, L. and Mazanti, R.
*Cancer Res., **69(18)**, 7235-7242 (2009)*

The multidrug resistance (MDR) phenotype is characterized by the overexpression of a few transport proteins at the plasma membrane level, one of which is the breast cancer resistance protein (BCRP). These proteins are expressed in excretory organs, in the placenta and blood-brain barrier, and are involved in the transport of drugs and endogenous compounds. Because some of these proteins are expressed in the mitochondria, this study was designed to determine whether BCRP is expressed at a mitochondrial level and to investigate its function in various MDR and parental drug–sensitive cell lines. By using Western blot analysis, immunofluorescence confocal and electron microscopy, flow cytometry analysis, and the BCRP (ABCG-2) small interfering RNA, these experiments showed that BCRP is expressed in the mitochondrial cristae, in which it is functionally active. Mitoxantrone accumulation was significantly reduced in mitochondria and in cells that overexpress BCRP, in comparison to parental drug–sensitive cells. The specific inhibitor of BCRP, fumitremorgin c, increased the accumulation of mitoxantrone significantly in comparison with basal conditions in both whole cells and in mitochondria of BCRP-overexpressing cell lines. In conclusion, this study shows that BCRP is overexpressed and functionally active in the mitochondria of MDR-positive cancer cell lines. However, its presence in the mitochondria of parental drug–sensitive cells suggests that BCRP can be involved in the physiology of cancer cells.

3.1324 Regulation of Cell Death by Recycling Endosomes and Golgi Membrane Dynamics via a Pathway Involving Src-family kinases, Cdc42 and Rab11a
Landry, M-L., Sicotte, A., Champagne, C. and Lavoie, J.N.
*Mol. Biol. Cell, **20**, 4091-4106 (2009)*

Actin dynamics and membrane trafficking influence cell commitment to programmed cell death through largely undefined mechanisms. To investigate how actin and recycling endosome (RE) trafficking can engage death signaling, we studied the death program induced by the adenovirus early region 4 open reading frame 4 (E4orf4) protein as a model. We found that in the early stages of E4orf4 expression, Src-family kinases (SFKs), Cdc42, and actin perturbed the organization of the endocytic recycling compartment and promoted the transport of REs to the Golgi apparatus, while inhibiting recycling of protein cargos to the plasma membrane. The resulting changes in Golgi membrane dynamics that relied on actin-regulated Rab11a membrane trafficking triggered scattering of Golgi membranes and contributed to the progression of cell death. A similar mobilization of RE traffic mediated by SFKs, Cdc42 and Rab11a also contributed to Golgi fragmentation and to cell death progression in response to staurosporine, in a caspase-independent manner. Collectively, these novel findings suggest that diversion of RE trafficking to the Golgi complex through a pathway involving SFKs, Cdc42, and Rab11a plays a general role in death signaling by mediating regulated changes in Golgi dynamics.

3.1325 **The Small Heat Shock Protein αB-Crystallin Is Secreted From Retinal Pigment Epithelial Cells ARPE in Culture**

Bhat, S.P., Gangalum, R.K. and Jing, Z.

Invest. Ophthalmol. Vis. Sci., ***50****, abstract 4183 (2009)*

Purpose: Elevated expression of the small heat shock protein, αB-crystallin (αB) has been associated with cancer, cardiomyopathies and a large number of neurodegenerative disorders including, Alzheimer's, Parkinson's, Alexander's diseases, multiple sclerosis and retinal degenerations. Interestingly, the expression of this protein in retinal pigment epithelium (RPE) and its presence in 'drusen'; has been implicated in age-related macular degeneration. In this investigation we have explored the status of αB in ARPE cells in culture with a focus on mechanistic understanding of how αB becomes associated with the 'drusen'. Methods: The association of αB with Golgi membranes was studied by sucrose density gradient fractionation and confocal microscopy. Protein transport was studied using various inhibitors. Exosomes were isolated from the culture medium by differential centrifugation, characterized by AChE assay and immuno-electron microscopy. Optiprep gradients were used to isolate lipid rafts from ARPE cells and characterized by the presence of established raft markers. Results: Using synchronized cultures we show that αB is a Golgi -associated protein in ARPE. We demonstrate that αB is secreted out of ARPE and that this secretion is not inhibited by classical protein transport inhibitors such as Brefeldin and Monensin, and glycosylation inhibitor, Tunicamycin and microtubule disrupting agent, Nocodazole. Examination of the culture medium reveals that αB is associated with exosomes and multivesicular bodies (MVB). Interestingly the secretion of this protein is inhibited by methyl α-cyclodextrin (MBC) suggesting lipid raft dependent exocytosis of vesicles containing αB. Conclusion: The small heat shock protein αB is secreted from ARPE cells via a non-conventional secretory pathway that involves the lipid rafts, and biogenesis and release of MVBs and exosomes. While these data point to an involvement of B with membrane trafficking, polarization and cellular secretion, they also suggest a possible route to the presence of this protein in the 'drusen'.

3.1326 **Omega-3 Protects Trabecular Meshwork From Metabolic Stress**

Beverley, R.M., Nolan, M.J., McCarty, R.D., Yue, B.Y.J.T., Qi, L.

Invest. Ophthalmol. Vis. Sci., ***50****, abstract 4866 (2009)*

Purpose: Supplemental dietary omega-3 fatty acid reduces IOP in rats and is neuroprotective against oxidative stress in retinal pigment epithelial cells. The omega-3 fatty acid docosahexaenoic acid (DHA) is an important omega-3 representing a large component of total acyl chains in nervous tissue and is concentrated in lipid rafts. The purpose of this study was to determine whether trabecular meshwork (TM) cell survival is influenced by DHA and the effects of DHA on caveolin/lipid rafts.
Methods: Primary cultures of human TM cells were pre-treated with 0.67, 2.01 and 6.03 µM DHA for 24 hrs and then challenged with metabolic stress using 1, 10 and 40 mM lactate for 3 hrs. Cell viability was determined. Lipid rafts in cell lysates were separated by an **Optiprep** density gradient (Sigma D1556). The preparation was centrifuged at 200,000 x g for 18 hrs; nine 1.0 ml fractions were pipetted from the top (lightest) to bottom (heaviest). Each fraction was analyzed for protein content, resolved by SDS polyacrylamide electrophoresis, and immunoblotted with anti-caveolin-1, anti-caveolin-2, and LRP-6 antibodies.
Results: There was a statistically significant dose dependent increase in cell survival of TM cells subjected to lactate stress following DHA treatment; 0.67 µM pretreatment was insignificant, 2.01 µM pretreatment was significant ($P<0.03$), and 6.03 µM pretreatment was highly significant ($P<0.001$). The distribution of 22-kDa caveolin-1 in DHA-treated TM cells was markedly increased in fractions 1, 2, 4, and 5 compared to controls. The distribution of caveolin-2 in DHA-treated cells was increased in fractions 1 and 2, presumably forming homo- and heterodimers with caveolin-1. LRP-6 was strongly upregulated in fractions 3, 4, 5, and 6 compared to controls.
Conclusions:DHA treatment increased cell viability and changed caveolin-1, caveolin-2, and LRP-6 density gradient distribution in TM cells subjected to metabolic stress. These results indicate that DHA modulates the distribution and content of caveolin/lipid rafts which may play an important role in TM cell viability and cell signaling.

3.1327 **Gamma Secretase and Trabecular Meshwork Stress**

McCarty, R.D., Nolan, M.J., Beverley, R.M., Quinn, K., Yue, B.Y.J.T., Samples, J.R. and Knepper, P.A.

Invest. Ophthalmol. Vis. Sci., ***50****, abstract 4870 (2009)*

Purpose: Primary open-angle glaucoma (POAG) and Alzheimer's disease share many similarities in pathogenic mechanisms. γ-secretase is a presenilin dependent enzyme which mediates the proteolytic cleavage of several transmembrane proteins. Examples of γ-secretase substrates include CD44 to form soluble sCD44, a potential biomarker of POAG, amyloid precursor to form amyloid β-peptide, a biomarker of Alzheimer's disease, LDL receptor-related protein, E-cadherin and Erb-4. The intracellular domains (ICD) of γ-secretase substrates are liberated and function as nuclear signals. The purpose of this study was to determine whether metabolic stress alters γ-secretase, β-secretase, and MT1-MMP in trabecular meshwork (TM) cells.
Methods: Human TM cells were grown in modified Eagle's medium containing 10% fetal bovine serum (FBS) until confluent, washed twice with PBS, and incubated in MEM containing 0.1% FBS with 1, 10, and 40 mM lactate for 3, 12, and 24 hrs. The media was aspirated; the cells were washed with cold PBS, subjected to lysis buffer (Sigma CS0750) containing 1% Triton X-100, and separated by an **Optiprep** density gradient (Sigma D1556). The preparation was centrifuged at 200,000 x g for 18 hrs; nine 1.0 ml fractions were pipetted from the top (lightest) to bottom (heaviest). Each fraction was analyzed for protein content, resolved by SDS polyacrylamide electrophoresis, and immunoblotted with anti-γ-secretase, β-secretase, MT1-MMP, and caveolin-1.
Results: The distribution of γ-secretase in lactate stressed TM cells was in fractions 3 through 9 and a decreased intensity was observed in fractions 6 through 9 compared to controls. In contrast, the distribution of β-secretase was observed in fractions 5 through 7 with a robust intensity (increased more than three-fold) in fractions 6 and 7 compared to controls. There was no demonstrable change in the distribution of MT1-MMP in metabolic stressed TM cells. The content of caveolin-1 was markedly decreased in fractions 4 and 5 compared to controls.
Conclusions: This is a first demonstration of γ- and β-secretase, and their modulation by metabolic stress, in TM cells. Both γ- and β-secretase are concentrated in caveolin lipid rafts, and the decrease in γ-secretase and increase in β-secretase may be important in the TM response to metabolic stress.

3.1328 Wnt Signaling and Trabecular Meshwork Lipid Rafts
Knepper, P.A., Beverley, R.M., McCarty, R.D., Nolan, M.J., Yue, B.Y.J.T. and Samples, J.R.
Invest. Ophthamol. Vis. Sci., ***50***, *abstract 4875 (2009)*

Purpose: Wnt proteins interact with Frizzled receptors and members of the low-density-lipoprotein receptor (LRP). LRP serves as an endocytosis receptor for α-2-macroglobulin, a mediator of retinal ganglion cell death in glaucoma and also for amyloid precursor protein, a mediator of selected cell death in Alzheimer's disease. Secreted frizzled-related protein-1 (sFRP-1) is an antagonist of Wnt signaling that causes a decreased outflow facility in ex vivo perfusion-of cultured human eyes. To explore possible signaling mechanisms by which sFRP-1 decreases outflow facility, we challenged trabecular meshwork (TM) cells with exogenous sFRP-1 and evaluated its effects on cell viability and its downstream effects on Wnt pathway of LRP-6 (canonical) and CD44 (non-canonical) content in caveolin/lipid rafts.
Methods: Human TM cells cultured in the presence 2.5, 5.0 and 10.0 ug of recombinant human sFRP-1 (R & D Systems) were tested for cell survival. Caveolin enriched lipid rafts were isolated using ice cold Triton X-100 and then separated on an **OptiPrep** density gradient (Sigma D1556). The preparation was centrifuged at 200,000 x g for 18 hrs; nine 1.0 ml fractions were pipetted from the top (lightest) to bottom (heaviest). Each fraction was analyzed for protein content, resolved by SDS polyacrylamide electrophoresis, and immunoblotted with anti-caveolin-1, CD44, and LRP-6 antibodies.
Results: There was a statistically significant ($P<0.001$) dose dependent decrease of cell viability in TM cells treated with sFRP-1. The distribution of 22-kDa caveolin-1 in sFRP-1 treated TM cells was in fractions 3 through 7 with the strongest intensity in fractions 5 and 6. The amount of caveolin-1 in sFRP-1 treated TM cells was considerably greater, approximately two-fold. The distribution of CD44 in sFRP-1 treated TM cells remained in fractions 1 through 9 but with an increased content of 32 kDa CD44 in fraction 5 compared to controls. The distribution of LRP-6 in sFRP-1 treated TM cells was in fractions 4 through 9 and decreased compared to control TM cells.
Conclusions: This is the first demonstration of the effects of exogenous sFRP-1 on the downstream signaling of caveolin, CD44 and LRP-6 in TM cells. sFRP-1 increased the cell caveolin-1 content, changed caveolin-1 density gradient distribution, decreased LRP-6 content and increased sCD44. These results indicate that caveolin-1, CD44 and/or LRP-6 are downstream effectors of sFRP-1.

3.1329 Proteomic analysis of *Legionella*-containing phagosomes isolated from *Dictyostelium*
Shevchuk, O., Batzillaa, C., Hägele, S., Kusch, H., Engelmann, S., Hecker, M., Haas, A., Heuner, K., Glöckner, G. and Steinert, M.
Int. J. Med. Microbiol., ***299***, *489-508 (2009)*

Legionella pneumophila, the agent of Legionnaires' disease, replicates intracellularly within specialized phagosomes of human macrophages and amoebae. In this study, we have developed a protocol for the isolation of *Legionella*-containing phagosomes from *Dictyostelium discoideum*. Cell fractionation, two-dimensional gel electrophoresis and MALDI-TOF MS combined with genomic data identified 157 phagosome host proteins. In addition to proteins with an evident role in phagosome maturation, we identified proteins for which a function remains to be elucidated. Possible interactions of coronin with cytosolic NADPH oxidase components and protein kinase C inhibitors which together may lead to an inhibition of phagosomal superoxide generation are discussed. Comparative proteomics of phagosomes containing highly virulent *L. pneumophila* Corby versus less virulent *L. hackeliae* revealed distinctive protein expression patterns, e.g., an abundance of RhoGDI in *L. hackeliae* degrading phagosomes versus little RhoGDI in *L. pneumophila* Corby replicative phagosomes. We present a kinetic dissection of phagosome maturation including the complex alterations of the phagosome protein composition. A reference flow chart suggests so far unrecognized consequences of infection for host cell physiology, actin degradation on phagosomes, and a putative cysteine proteinase inhibitor interference with lysosomal enzyme sorting and activation processes.

3.1330 Formation and function of hepatitis C virus replication complexes require residues in the carboxy-terminal domain of NS4B protein

Aligo, J., Jia, S., Manna, D. and Konan, K.V.
Virology, ***393***, *68-83 (2009)*

During replication, hepatitis C virus (HCV) NS4B protein rearranges intracellular membranes to form foci, or the web, the putative site for HCV replication. To understand the role of the C-terminal domain (CTD) in NS4B function, mutations were introduced into NS4B alone or in the context of HCV polyprotein. First, we show that the CTD is required for NS4B-induced web structure, but it is not sufficient to form the web nor is it required for NS4B membrane association. Interestingly, all the mutations introduced into the CTD impeded HCV genome replication, but only two resulted in a disruption of NS4B foci. Further, we found that NS4B interacts with NS3 and NS5A, and that mutations causing NS4B mislocalization have a similar effect on these proteins. Finally, we show that the redistribution of Rab5 to NS4B foci requires an intact CTD, suggesting that Rab5 facilitates NS4B foci formation through interaction with the CTD.

3.1331 Caveolae Contribute to the Apoptosis Resistance Induced by the α_{1A}-Adrenoceptor in Androgen-Independent Prostate Cancer Cells

Katsogiannou, M., El Boustany, C., Gackiere, F., Delcourt, P., Athias, A., Mariot, P., Dewailly, E., Jouuy, N., Lamaze, C., Bidaux, G., Mauroy, B., Prevarskay, N. and Slomianny, C.
PloSOne, ***4(9)***, *e7068 (2009)*

Background
During androgen ablation prostate cancer cells' growth and survival become independent of normal regulatory mechanisms. These androgen-independent cells acquire the remarkable ability to adapt to the surrounding microenvironment whose factors, such as neurotransmitters, influence their survival. Although findings are becoming evident about the expression of α_{1A}-adrenoceptors in prostate cancer epithelial cells, their exact functional role in androgen-independent cells has yet to be established. Previous work has demonstrated that membrane lipid rafts associated with key signalling proteins mediate growth and survival signalling pathways in prostate cancer cells.
Methodology/Principal Findings
In order to analyze the membrane topology of the α_{1A}-adrenoceptor we explored its presence by a biochemical approach in purified detergent resistant membrane fractions of the androgen-independent prostate cancer cell line DU145. Electron microscopy observations demonstrated the colocalisation of the α_{1A}-adrenoceptor with caveolin-1, the major protein component of caveolae. In addition, we showed that agonist stimulation of the α_{1A}-adrenoceptor induced resistance to thapsigargin-induced apoptosis and that caveolin-1 was necessary for this process. Further, immunohistofluorescence revealed the relation between high levels of α_{1A}-adrenoceptor and caveolin-1 expression with advanced stage prostate cancer. We also show by immunoblotting that the TG-induced apoptosis resistance described in DU145 cells is mediated by extracellular signal-regulated kinases (ERK).
Conclusions/Significance
In conclusion, we propose that α_{1A}-adrenoceptor stimulation in androgen-independent prostate cancer cells *via* caveolae constitutes one of the mechanisms contributing to their protection from TG-induced apoptosis.

3.1332 High extracellular glucose inhibits exocytosis through disruption of syntaxin 1A-containing lipid rafts

Somanath, S., Barg, S., Marshall, C., Silwood, C.J. and Turner, M.D.
Biochem. Biophys. Res. Comm., ***389***, *241-246 (2009)*

Diabetes is characterized by high blood glucose which eventually impairs the secretion of insulin. Glucose directly affects cholesterol biosynthesis and may in turn affect cellular structures that depend on the sterol, including lipid rafts that help organize the secretory apparatus. Here, we investigated the long-term effects of glucose upon lipid rafts and secretory granule dynamics in pancreatic β-cells. Raft fractions, identified by the presence of GM1 and flotillin, contained characteristically high levels of cholesterol and syntaxin 1A, the t-SNARE which tethers granules to the plasma membrane. Seventy-two hours exposure to 28 mM glucose resulted in ~30% reduction in membrane cholesterol, with consequent redistribution of raft markers and syntaxin 1A throughout the plasma membrane. Live cell imaging indicated loss of syntaxin 1A from granule docking sites, and fewer docked granules. In conclusion, glucose-mediated inhibition of cholesterol biosynthesis perturbs lipid raft stability, resulting in a loss of syntaxin 1A from granule docking sites and inhibition of insulin secretion.

3.1333 Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion

DeVay, R.M., Dominguez-Ramirez, L., Lackner, L.L., Hoppins, S., Stahlberg, H. and Nunnari, J.
J. Cell Biol., ***186(6)***, *793-803 (2009)*

Two dynamin-related protein (DRP) families are essential for fusion of the outer and inner mitochondrial membranes, Fzo1 (yeast)/Mfn1/Mfn2 (mammals) and Mgm1 (yeast)/Opa1 (mammals), respectively. Fzo1/Mfns possess two medial transmembrane domains, which place their critical GTPase and coiled-coil domains in the cytosol. In contrast, Mgm1/Opa1 are present in cells as long (l) isoforms that are anchored via the N terminus to the inner membrane, and short (s) isoforms were predicted to be soluble in the intermembrane space. We addressed the roles of Mgm1 isoforms and how DRPs function in membrane fusion. Our analysis indicates that in the absence of a membrane, l- and s-Mgm1 both exist as inactive GTPase monomers, but that together in trans they form a functional dimer in a cardiolipin-dependent manner that is the building block for higher-order assemblies.

3.1334 Dynamic clustering and dispersion of lipid rafts contribute to fusion competence of myogenic cells

Mukai, A., Kurisaki, T., Sato, S.B., Kobayashi, T., Kondoh, G. and Hashimoto, N.
Exp. Cell Res., ***315***, *3052-3063 (2009)*

Recent research indicates that the leading edge of lamellipodia of myogenic cells (myoblasts and myotubes) contains presumptive fusion sites, yet the mechanisms that render the plasma membrane fusion-competent remain largely unknown. Here we show that dynamic clustering and dispersion of lipid rafts contribute to both cell adhesion and plasma membrane union during myogenic cell fusion. Adhesion-complex proteins including M-cadherin, β-catenin, and p120-catenin accumulated at the leading edge of lamellipodia, which contains the presumptive fusion sites of the plasma membrane, in a lipid raft-dependent fashion prior to cell contact. In addition, disruption of lipid rafts by cholesterol depletion directly prevented the membrane union of myogenic cell fusion. Time-lapse recording showed that lipid rafts were laterally dispersed from the center of the lamellipodia prior to membrane fusion. Adhesion proteins that had accumulated at lipid rafts were also removed from the presumptive fusion sites when lipid rafts were laterally dispersed. The resultant lipid raft- and adhesion complex-free area at the leading edge fused with the opposing plasma membrane. These results demonstrate a key role for dynamic clustering/dispersion of lipid rafts in establishing fusion-competent sites of the myogenic cell membrane, providing a novel mechanistic insight into the regulation of myogenic cell fusion.

3.1335 Phosphotyrosine protein dynamics in cell membrane rafts of sphingosine-1-phosphate-stimulated human endothelium: Role in barrier enhancement

Zhao, J., Singleton, P.A., Brown, M.E., Dudek, S.M. and Garcia, J.G.N.
Cellular Signalling, ***21***, *1945-1960 (2009)*

Sphingosine-1-phosphate (S1P), a lipid growth factor, is critical to the maintenance and enhancement of vascular barrier function via processes highly dependent upon cell membrane raft-mediated signaling events. Anti-phosphotyrosine 2 dimensional gel electrophoresis (2-DE) immunoblots confirmed that disruption of membrane raft formation (via methyl-β-cyclodextrin) inhibits S1P-induced protein tyrosine

phosphorylation. To explore S1P-induced dynamic changes in membrane rafts, we used 2-D techniques to define proteins within detergent-resistant cell membrane rafts which are differentially expressed in S1P-challenged (1 μM, 5 min) human pulmonary artery endothelial cells (EC), with 57 protein spots exhibiting > 3-fold change. S1P induced the recruitment of over 20 cell membrane raft proteins exhibiting increasing levels of tyrosine phosphorylation including known barrier-regulatory proteins such as focal adhesion kinase (FAK), cortactin, p85α phosphatidylinositol 3-kinase (p85αPI3K), myosin light chain kinase (nmMLCK), filamin A/C, and the non-receptor tyrosine kinase, c-Abl. Reduced expression of either FAK, MLCK, cortactin, filamin A or filamin C by siRNA transfection significantly attenuated S1P-induced EC barrier enhancement. Furthermore, S1P induced cell membrane raft components, p-caveolin-1 and glycosphingolipid (GM1), to the plasma membrane and enhanced co-localization of membrane rafts with p-caveolin-1 and p-nmMLCK. These results suggest that S1P induces both the tyrosine phosphorylation and recruitment of key actin cytoskeletal proteins to membrane rafts, resulting in enhanced human EC barrier function.

3.1336 A Novel Role for Nutrition in the Alteration of Functional Microdomains on the Cell Surface
Kim, W., Chapkin, R.S., Barhoumi, R. and Ma, D.W.L.
Methods in Mol. Biol., ***579***, *261-270 (2009)*

Membrane rafts are ordered microdomains of the plasma membrane consisting of cholesterol, sphingolipids, and saturated fatty acids which appear to regulate many cellular signaling pathways. One such type of membrane raft is caveolae, which are cave-like invaginations of the plasma membrane. Interestingly, changes in the acyl composition of cellular membranes have been shown to alter the specific localization of membrane raft associated proteins and their function. This is noteworthy because modification of membrane acyl composition is readily accomplished through changes in dietary fat composition. Here we describe a common approach used to fractionate cell membranes to obtain an enriched preparation of caveolae and gas chromatographic techniques to determine fatty acyl composition. In addition, methods used to visualize and quantify lipid rafts using a fluorescent probe Laurdan in living cells will also be described.

3.1337 Lipid Raft-Redox Signaling Platforms in Plasma Membrane
Yi, F., Jin, S. and Li, P-L.
Methods in Mol. Biol., ***580***, *93-107 (2009)*

Membrane lipid rafts (LRs) have been demonstrated to be importantly involved in transmembrane signaling in a variety of mammalian cells. Many receptors can be aggregated within the LR clusters to form signaling platforms. Currently, LRs were reported to be clustered to aggregate, recruit, and assemble NADPH oxidase subunits and related proteins in various cells in response to various stimuli, forming redox signaling platforms. These LR signaling platforms may play important roles in the regulation of cellular activity and cell function, and also in the development of cell dysfunction or injury associated with various pathological stimuli. This LRs clustering-mediated mechanism is considered to take a center stage in redox signaling associated with death receptors. In this chapter, some basic methods and procedures for characterization of LR-redox signaling platforms formation and for determination of the function of these signaling platforms are described in detail, which include identification of LR-redox signaling platforms in cell membrane by using fluorescent or confocal microscopy of LR-redox signaling platforms and fluorescent resonance energy transfer analysis, isolation of LR-redox signaling platforms by flotation of detergent-resistant membranes, and function measurement of LR-redox signaling platforms by electron spin resonance spectroscopy. It is expected that information provided here will help readers to design necessary experiments in their studies on LR signaling platforms and redox regulation of cell function.

3.1338 Hsp90 Co-localizes with Rab-GDI-1 and Regulates Agonist-induced Amylase Release in AR42J Cells
Raffaniello, R., Fedorova, D., Ip, D. and Rafiq, S.
Cell. Physiol. Biochem., ***24(5-6)***, *369-378 (2009)*

Rab proteins are small GTPases required for vesicle trafficking through the secretory and endocytic pathways. Rab GDP-dissociation inhibitor (rab-GDI) regulates Rab protein function and localization by maintaining Rab proteins in the GDP-bound conformation. Two isoforms of rab-GDI are present in most mammalian cells: GDI-1 and GDI-2. It has recently been demonstrated that a Heat shock protein 90 (Hsp90) chaperone complex regulates the interactions between Rab proteins and Rab-GDI-1. The AR42J cell line is derived from rat pancreatic exocrine tumor cells and develops an acinar-like phenotype when

treated with dexamethasone (Dex). The aim of the present study was to examine the expression of rab-GDI isoforms and Hsp90 in AR42J cells in the presence or absence of Dex. Rab-GDI:Hsp90 interactions were also examined. Both rab-GDI isoforms were detected in AR42J cells by immunoblotting. In Dex-treated cells, quantitative immunoblotting revealed that rab-GDI-1 expression increased by 28%, although this change was not statistically significant. Rab-GDI-2 levels were unaltered by Dex treatment. Approximately 21% rab-GDI-1 was membrane associated, whereas rab-GDI-2 was exclusively cytosolic. Dex treatment did not affect the subcellular distribution of rab-GDI isoforms. Hsp90 was present in the cytosolic and membrane fractions of AR42J cells and co-immunoprecipitated with cytosolic rab-GDI-1. Moreover, density gradient centrifugation of AR42J cell membranes revealed that Hsp90 and rab-GDI-1 co-localize on low- and high-density membrane fractions, including amylase-containing secretory granules. The Hsp90 inhibitor, geldanamycin, inhibited CCK-8-induced amylase release from these cells in a dose-dependent manner. Our results indicate that as AR42J cells differentiate into acinar-like cells, rab-GDI isoform expression and localization is not significantly altered. Moreover, our findings suggest that Hsp90 regulates agonist-induced secretion in exocrine cells by interacting with rab-GDI-1.

3.1339 The cytosolic domain of PEX3, a protein involved in the biogenesis of peroxisomes, binds membrane lipids

Pinto, M.P., Grou, C.P., Fransen, M., Sa-Miranda, C. And Azevedo, J.E.
*Biochim. Biophys. Acta, **1793**, 1669-1675 (2009)*

According to current models, most newly synthesized peroxisomal intrinsic membrane proteins are recognized in the cytosol and targeted to the peroxisomal membrane by PEX19. At the organelle membrane the PEX19-cargo protein complex interacts with PEX3, a protein believed to possess only one transmembrane domain and exposing the majority of its polypeptide chain into the cytosol. In agreement with this topological model, a recombinant protein comprising the cytosolic domain of PEX3 can be purified in a soluble and monomeric form in the absence of detergents or other solubilizing agents. Here, we show that this recombinant protein actually precipitates when incubated with mild detergents, suggesting that this domain of PEX3 interacts with amphipathic molecules. Following this observation, we tested this recombinant protein in lipid-binding assays and found that it interacts strongly with liposomes inducing their flocculation or even partial solubilization. The implications of these findings are discussed.

3.1340 An involvement of yeast peroxisomal channels in transmembrane transfer of glyoxylate cycle intermediates

Antonenkov, V.D., Mindhoff, S., Grunau, S., Erdmann, R. And Hiltunen, J.K.
*Int. J. Biochem. Cell. Biol., **41(2)**, 2546-2554 (2009)*

The separate localization of glyoxylate cycle enzymes in the peroxisomes and the cytosol of the yeast *Saccharomyces cerevisiae* indicates that the peroxisomal membrane must permit the flow of metabolites between the two compartments. The transfer of these metabolites may require peroxisomal membrane channel(s). We used an electrophysiological approach (reconstitution assay in lipid bilayers) to assess the ability of peroxisomal membrane channels to conduct different solutes including metabolites of the glyoxylate cycle. At least two distinct channel-forming activities were detected in peroxisomal preparations. One of these activities was highly inducible by dithiothreitol and showed large-amplitude current increments when 1 M KCl was used as a bath solution. Single-channel analysis revealed that the inducible channel is anion-selective (P_{Cl^-}/P_{K^+}=2.6; $P_{citrate}/P_{K^+}$=1.6) and displays flickering at holding potentials over ±30 mV directed upward or downward relative to the main open state of the channel. The channel inducible by DTT facilitates the transfer of solutes with a molecular mass up to 400 Da, sufficient to allow the transmembrane trafficking of glyoxylate cycle intermediates between the peroxisomal lumen and the cytoplasm.

3.1341 *Cryptococcus neoformans* cryoultramicrotomy and vesicle fractionation reveals an intimate association between membrane lipids and glucuronoxylomannan

Oliveira, D.., Nimrichter, L., Miranda, K., Frases, S., Faull, K.F., Casadevall, A. And Roddrigues, M.L.
*Fungal Genetics and Biology, **46**, 956-963 (2009)*

Cryptococcus neoformans is an encapsulated pathogenic fungus. The cryptococcal capsule is composed of polysaccharides and is necessary for virulence. It has been previously reported that glucuronoxylomannan (GXM), the major capsular component, is synthesized in cytoplasmic compartments and transported to the extracellular space in vesicles, but knowledge on the organelles involved in polysaccharide synthesis and traffic is extremely limited. In this paper we report the GXM distribution in *C. neoformans* cells sectioned

by cryoultramicrotomy and visualized by transmission electron microscopy (TEM) and polysaccharide immunogold staining. Cryosections of fungal cells showed high preservation of intracellular organelles and cell wall structure. Incubation of cryosections with an antibody to GXM revealed that cytoplasmic structures associated to vesicular compartments and reticular membranes are in close proximity to the polysaccharide. GXM was generally found in association with the membrane of intracellular compartments and within different layers of the cell wall. Analysis of extracellular fractions from cryptococcal supernatants by transmission electron microscopy in combination with serologic, chromatographic and spectroscopic methods revealed fractions containing GXM and lipids. These results indicate an intimate association of GXM and lipids in both intracellular and extracellular spaces consistent with polysaccharide synthesis and transport in membrane-associated structures.

3.1342 Eicosanoid Release Is Increased by Membrane Destabilization and CFTR Inhibition in Calu-3 Cells
Borot, F., Vieu, D-L., Faure, G., Fritsch, J., Colas, J., Moriceau, S., Baudouin-Legros, M., Brouillard, F., Ayala-Sanmartin, J., Touqui, L., Chanson, M., Edelman, A. and Ollero, M.
PloSOne, ***4(10)***, *e7116 (2009)*

The antiinflammatory protein annexin-1 (ANXA1) and the adaptor S100A10 (p11), inhibit cytosolic phospholipase A2 (cPLA2α) by direct interaction. Since the latter is responsible for the cleavage of arachidonic acid at membrane phospholipids, all three proteins modulate eicosanoid production. We have previously shown the association of ANXA1 expression with that of CFTR, the multifactorial protein mutated in cystic fibrosis. This could in part account for the abnormal inflammatory status characteristic of this disease. We postulated that CFTR participates in the regulation of eicosanoid release by direct interaction with a complex containing ANXA1, p11 and cPLA2α. We first analyzed by plasmon surface resonance the in vitro binding of CFTR to the three proteins. A significant interaction between p11 and the NBD1 domain of CFTR was found. We observed in Calu-3 cells a rapid and partial redistribution of all four proteins in detergent resistant membranes (DRM) induced by TNF-α. This was concomitant with increased IL-8 synthesis and cPLA2α activation, ultimately resulting in eicosanoid (PGE2 and LTB4) overproduction. DRM destabilizing agent methyl-β-cyclodextrin induced further cPLA2α activation and eicosanoid release, but inhibited IL-8 synthesis. We tested in parallel the effect of short exposure of cells to CFTR inhibitors Inh172 and Gly-101. Both inhibitors induced a rapid increase in eicosanoid production. Longer exposure to Inh172 did not increase further eicosanoid release, but inhibited TNF-α-induced relocalization to DRM. These results show that (i) CFTR may form a complex with cPLA2α and ANXA1 *via* interaction with p11, (ii) CFTR inhibition and DRM disruption induce eicosanoid synthesis, and (iii) suggest that the putative cPLA2/ANXA1/p11/CFTR complex may participate in the modulation of the TNF-α-induced production of eicosanoids, pointing to the importance of membrane composition and CFTR function in the regulation of inflammation mediator synthesis.

3.1343 ABC Transporter Pdr10 Regulates the Membrane Microenvironment of Pdr12 in Saccharomyces cerevisiae
Rockwell, N.C., Wolfger, H., Kuchler, K. and Thorner, J.
J. Membrane Biol., ***229***, *27-52 (2009)*

The eukaryotic plasma membrane exhibits both asymmetric distribution of lipids between the inner and the outer leaflet and lateral segregation of membrane components within the plane of the bilayer. In budding yeast (Saccharomyces cerevisiae), maintenance of leaflet asymmetry requires P-type ATPases, which are proposed to act as inward-directed lipid translocases (Dnf1, Dnf2, and the associated protein Lem3), and ATP-binding cassette (ABC) transporters, which are proposed to act as outward-directed lipid translocases (Pdr5 and Yor1). The S. cerevisiae genome encodes two other Pdr5-related ABC transporters: Pdr10 (67% identity) and Pdr15 (75% identity). We report the first analysis of Pdr10 localization and function. A Pdr10-GFP chimera was located in discrete puncta in the plasma membrane and was found in the detergent-resistant membrane fraction. Compared to control cells, a pdr10Δ mutant was resistant to sorbate but hypersensitive to the chitin-binding agent Calcofluor White. Calcofluor sensitivity was attributable to a partial defect in endocytosis of the chitin synthase Chs3, while sorbate resistance was attributable to accumulation of a higher than normal level of the sorbate exporter Pdr12. Epistasis analysis indicated that Pdr10 function requires Pdr5, Pdr12, Lem3, and mature sphingolipids. Strikingly, Pdr12 was shifted to the detergent-resistant membrane fraction in pdr10Δ cells. Pdr10 therefore acts as a negative regulator for incorporation of Pdr12 into detergent-resistant membranes, a novel role for members of the ABC transporter superfamily.

3.1344 Solubilization, purification, and reconstitution of α2β1 isozyme of Na+/K+-ATPase from caveolae of pulmonary smooth muscle plasma membrane: comparative studies with DHPC, C12E8, and Triton X-100

Ghosh, B., Chakraborti, T., Kar, P., Dey, K. and Chakraborti, S.
Mol. Cell. Biochem., ***323***, *169-184 (2009)*

We identified α2, α1, and β1 isoforms of Na+/K+-ATPase in caveolae vesicles of bovine pulmonary smooth muscle plasma membrane. The biochemical and biophysical characteristics of the α2β1 isozyme of Na+/K+-ATPase from caveolae vesicles were studied during solubilization and purification using the detergents 1,2-heptanoyl-sn-phosphatidylcholine (DHPC), poly(oxy-ethylene)8-lauryl ether (C12E8), and Triton X-100, and reconstitution with the phospholipid dioleoyl-phosphatidylcholine (DOPC). DHPC was determined to be superior to C12E8, whereas C12E8 was better than Triton X-100 in the active enzyme yields and specific activity. Fluorescence studies with DHPC-purified α2β1 isozyme of Na+/K+-ATPase elicited higher E1Na−E2 K transition compared with that of the C12E8- and Triton X-100-purified enzyme. The rate of Na+ efflux in DHPC–DOPC-reconstituted isozyme was higher compared to the C12E8–DOPC- and Triton X100–DOPC-reconstituted enzyme. Circular dichroism analysis suggests that the DHPC-purified α2β1 isozyme of Na+/K+-ATPase possessed more organized secondary structure compared to the C12E8- and Triton X-100-purified isozyme.

3.1345 Localisation of endothelin B receptor variants to plasma membrane microdomains and its effects on downstream signalling

Grossmann, S.., Higashiyama, S., Oksche, A., Schaefer, M. and Tannert, A.
Mol. Membrane Biol., ***26(5-7)***, *279-292 (2009)*

The endothelin B (ET_B) receptor can undergo a proteolytic cleavage resulting in an unglycosylated N-terminally truncated receptor. We investigated whether ET_B receptor processing affects caveolar localisation and mitogenic signalling. Distinct subcellular localisations of ET_B receptor constructs and epidermal growth factor (EGF) receptor ligands were analysed performing detergent-free caveolae preparations and total internal reflection fluorescence microscopy. ET_B receptor-induced transactivation of the EGF receptor and its downstream signalling was investigated performing shedding assays and ERK1/2 phosphorylation analyses. In COS7 cells, the N-terminally truncated but not the full-length or glycosylation-deficient ET_B receptor localised to caveolae. In caveolae-free HEK293 cells, only ET_B receptor constructs fused to caveolin-2 localised to membrane microdomains. A caveolar accumulation of the ET_B receptor disfavoured EGF receptor ligand shedding. Nonetheless, the activation of ERK1/2 was efficient and long-lasting. In HEK293 cells, the shedding activity was also impaired by N-terminal truncation. The subsequent ERK1/2 phosphorylation was long-lasting only for the full-length ET_B receptor. We conclude that the ET_B receptor localisation might depend on the presence of caveolae within the cell investigated. The data further suggest that caveolar enrichment of ET_B receptors does not facilitate the release of EGF receptor ligands. However, independent of their localisation, ET_B receptors are able to induce an ERK1/2 phosphorylation.

3.1346 Functions of lipid raft membrane microdomains at the blood–brain barrier

Dodelet-Devillers, A., cayrol, R., van Horssen, J., Haqqani, A.S., de Vries, H.E., Engelhardt, B., Greenwood, J. and Prat, A.
J. Mol. Med., ***87***, *765-774 (2009)*

The blood–brain barrier (BBB) is a highly specialized structural and functional component of the central nervous system that separates the circulating blood from the brain and spinal cord parenchyma. Brain endothelial cells (BECs) that primarily constitute the BBB are tightly interconnected by multiprotein complexes, the adherens junctions and the tight junctions, thereby creating a highly restrictive cellular barrier. Lipid-enriched membrane microdomain compartmentalization is an inherent property of BECs and allows for the apicobasal polarity of brain endothelium, temporal and spatial coordination of cell signaling events, and actin remodeling. In this manuscript, we review the role of membrane microdomains, in particular lipid rafts, in the BBB under physiological conditions and during leukocyte transmigration/diapedesis. Furthermore, we propose a classification of endothelial membrane microdomains based on their function, or at least on the function ascribed to the molecules included in such heterogeneous rafts: (1) rafts associated with interendothelial junctions and adhesion of BECs to basal lamina (*scaffolding rafts*); (2) rafts involved in immune cell adhesion and migration across brain endothelium (*adhesion rafts*); (3) rafts associated with transendothelial transport of nutrients and ions (*transporter rafts*).

3.1347 Lipid microdomain polarization is required for NADPH oxidase-dependent ROS signaling in Picea meyeri pollen tube tip growth
Liu, P., Li, R-L., Wang, Q-L., Niehaus, K., Baluska, F., Samaj, J. And Lin, J-X.
Plant J., ***60(2)***, *303-313 (2009)*

The polarization of sterol-enriched lipid microdomains has been linked to morphogenesis and cell movement in diverse cell types. Recent biochemical evidence has confirmed the presence of lipid microdomains in plant cells; however, direct evidence for a functional link between these microdomains and plant cell growth is still lacking. Here, we reported the involvement of lipid microdomains in NADPH oxidase (NOX)-dependent reactive oxygen species (ROS) signaling in Picea meyeri pollen tube growth. Staining with di-4-ANEPPDHQ or filipin revealed that sterol-enriched microdomains were polarized to the growing tip of the pollen tube. Sterol sequestration with filipin disrupted membrane microdomain polarization, depressed tip-based ROS formation, dissipated tip-focused cytosolic Ca2+ gradient and thereby arrested tip growth. NOX clustered at the growing tip, and corresponded with the ordered membrane domains. Immunoblot analysis and native gel assays demonstrated that NOX was partially associated with detergent-resistant membranes and, furthermore, that NOX in a sterol-dependent fashion depends on membrane microdomains for its enzymatic activity. In addition, in vivo time-lapse imaging revealed the coexistence of a steep tip-high apical ROS gradient and subapical ROS production, highlighting the reported signaling role for ROS in polar cell growth. Our results suggest that the polarization of lipid microdomains to the apical plasma membrane, and the inclusion of NOX into these domains, contribute, at least in part, to the ability to grow in a highly polarized manner to form pollen tubes.

3.1348 Hitchhiking of Cu/Zn Superoxide Dismutase to Peroxisomes - Evidence for a Natural Piggyback Import Mechanism in Mammals
Islinger, M., Li, K.W., Seitz, J., Völkl, A. And Lüers, G.H.
Traffic, ***10***, *1711-1721 (2009)*

Most newly synthesized peroxisomal proteins are imported in a receptor-mediated fashion, depending on the interaction of a peroxisomal targeting signal (PTS) with its cognate targeting receptor Pex5 or Pex7 located in the cytoplasm. Apart from this classic mechanism, heterologous protein complexes that have been proposed more than a decade ago are also to be imported into peroxisomes. However, it remains still unclear if this so-called piggyback import is of physiological relevance in mammals. Here, we show that Cu/Zn superoxide dismutase 1 (SOD1), an enzyme without an endogenous PTS, is targeted to peroxisomes using its physiological interaction partner 'copper chaperone of SOD1' (CCS) as a shuttle. Both proteins have been identified as peroxisomal constituents by 2D-liquid chromatography mass spectrometry of isolated rat liver peroxisomes. Yet, while a major fraction of CCS was imported into peroxisomes in a PTS1-dependent fashion in CHO cells, overexpressed SOD1 remained in the cytoplasm. However, increasing the concentrations of both CCS and SOD1 led to an enrichment of SOD1 in peroxisomes. In contrast, CCS-mediated SOD1 import into peroxisomes was abolished by deletion of the SOD domain of CCS, which is required for heterodimer formation. SOD1/CCS co-import is the first demonstration of a physiologically relevant piggyback import into mammalian peroxisomes.

3.1349 Urokinase-receptor-mediated phenotypic changes in vascular smooth muscle cells require the involvement of membrane rafts
Kiyan, J., Smith, G., Haller, H. And Dumler, I.
Biochem. J., ***423***, *343-351 (2009)*

The cholesterol-enriched membrane microdomains lipid rafts play a key role in cell activation by recruiting and excluding specific signalling components of cell-surface receptors upon receptor engagement. Our previous studies have demonstrated that the GPI (glycosylphosphatidylinositol)-linked uPAR [uPA (urokinase-type plasminogen activator) receptor], which can be found in lipid rafts and in non-raft fractions, can mediate the differentiation of VSMCs (vascular smooth muscle cells) towards a pathophysiological de-differentiated phenotype. However, the mechanism by which uPAR and its ligand uPA regulate VSMC phenotypic changes is not known. In the present study, we provide evidence that the molecular machinery of uPAR-mediated VSMC differentiation employs lipid rafts. We show that the disruption of rafts in VSMCs by membrane cholesterol depletion using MCD (methyl-β-cyclodextrin) or filipin leads to the up-regulation of uPAR and cell de-differentiation. uPAR silencing by means of interfering RNA resulted in an increased expression of contractile proteins. Consequently, disruption of

lipid rafts impaired the expression of these proteins and transcriptional activity of related genes. We provide evidence that this effect was mediated by uPAR. Similar effects were observed in VSMCs isolated from *Cav1*$^{-/-}$ (caveolin-1-deficient) mice. Despite the level of uPAR being significantly higher after the disruption of the rafts, uPA/uPAR-dependent cell migration was impaired. However, caveolin-1 deficiency impaired only uPAR-dependent cell proliferation, whereas cell migration was strongly up-regulated in these cells. Our results provide evidence that rafts are required in the regulation of uPAR-mediated VSMC phenotypic modulations. These findings suggest further that, in the context of uPA/uPAR-dependent processes, caveolae-associated and non-associated rafts represent different signalling membrane domains.

3.1350 Analysis of the dual function of the ESCRT-III protein Snf7 in endocytic trafficking and in gene expression
Weiss, P., Huppert, S. and Kölling, R.
Biochem. J., ***424***, *89-97 (2009)*

ESCRT (endosomal sorting complex required for transport)-III mediates the budding and scission of intralumenal vesicles into multivesicular endosomes in yeast. For the main ESCRT-III subunit Snf7, an additional role in activation of the transcription factor Rim101 (the 'Rim pathway') is now also firmly established. In the present study, we investigate how these two Snf7 functions are related to each other. By generating *SNF7* mutations that severely affect endocytic trafficking, but leave the Rim pathway function intact, we show that the two functions of *SNF7* can be separated genetically. We analysed in detail how the *SNF7* mutations affect the interaction of Snf7 with its various binding partners. Although the interactions with proteins Rim13 and Rim20, necessary for the Rim-pathway-related functions, were not altered by the mutations, there was a strong effect on interactions with components of the ESCRT pathway. The interactions, as measured by co-immunoprecipitation, with the ESCRT-III subunits Vps20 and Vps24 were strongly increased by the mutations, whereas the interactions with proteins Vps4 and Bro1, acting downstream of ESCRT-III, were reduced. As Vps4 is required for disassembly of ESCRT-III these results suggest that ESCRT-III is more stable in our *SNF7* mutants. In line with this notion, a higher fraction of mutant Snf7 protein was detected at the membrane. Upon a shift to alkaline pH, a stronger binding signal for virtually all interaction partners, except Vps4, was observed. This indicates that the ESCRT network at the endosomal membrane is more extensive under these conditions.

3.1351 Missense mutations in the SH3TC2 protein causing Charcot-Marie-Tooth disease type 4C affect its localization in the plasma membrane and endocytic pathway
Lupo, V., Galindo, M.I., Martinez-Rubio, D., Sevilla, T., Vilchez, J.J., Palau, F. and Espinos, C.
Hum. Mol. Genet., ***18(23)***, *4603-4614 (2009)*

Mutations in *SH3TC2* (*KIAA1985*) cause Charcot-Marie-Tooth disease (CMT) type 4C, a demyelinating inherited neuropathy characterized by early-onset and scoliosis. Here we demonstrate that the SH3TC2 protein is present in several components of the endocytic pathway including early endosomes, late endosomes and clathrin-coated vesicles close to the *trans*-Golgi network and in the plasma membrane. Myristoylation of SH3TC2 in glycine 2 is necessary but not sufficient for the proper location of the protein in the cell membranes. In addition to myristoylation, correct anchoring also needs the presence of SH3 and TPR domains. Mutations that cause a stop codon and produce premature truncations that remove most of the TPR domains are expressed as the wild-type protein. In contrast, missense mutations in or around the region of the first-TPR domain are absent from early endosomes, reduced in plasma membrane and late endosomes and are variably present in clathrin-coated vesicles. Our findings suggest that the endocytic and membrane trafficking pathway is involved in the pathogenesis of CMT4C disease. We postulate that missense mutations of *SH3TC2* could impair communication between the Schwann cell and the axon causing an abnormal myelin formation.

3.1352 Motor Protein–Dependent Membrane Trafficking of KCl Cotransporter-4 Is Important for Cancer Cell Invasion
Chen, Y-F., Chou, C-Y., Wilkins, R.J., Ellory, J.C., Mount, D.B. and Shen, M-R.
Cancer Res., ***69(22)***, *8585-8593 (2009)*

The KCl cotransporter (KCC) is a major determinant of osmotic homeostasis and plays an emerging role in tumor biology. This study stresses the important role of KCC4 in tumor malignant behavior. Real-time reverse transcription-PCR on samples collected by laser microdissection and immunofluorescent stainings with different KCC isoform antibodies indicate that KCC4 is abundant in metastatic cervical and ovarian cancer tissues. Insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) stimulate KCC4

recruitment from a presumably inactive cytoplasmic pool of endoplasmic reticulum and Golgi to plasma membrane along actin cytoskeleton that is significantly inhibited by LY294002 and wortmannin. Throughout the trafficking process, KCC4 is incorporated into lipid rafts that function as a platform for the association between KCC4 and myosin Va, an actin-dependent motor protein. KCC4 and ezrin, a membrane cytoskeleton linker, colocalize at lamellipodia of migratory cancer cells. Interference with KCC activity by either an inhibitor or a dominant-negative loss-of-function mutant profoundly suppressed the IGF-I–induced membrane trafficking of KCC4 and the structural interaction between KCC4 and ezrin near the cell surface. Endogenous cancer cell invasiveness was significantly attenuated by small interfering RNA targeting KCC4, and the residual invasiveness was much less sensitive to IGF-I or EGF stimulation. In the metastatic cancer tissues, KCC4 colocalizes with IGF-I or EGF, indicating a likely *in vivo* stimulation of KCC4 function by growth factors. Thus, blockade of KCC4 trafficking and surface expression may provide a potential target for the prevention of IGF-I– or EGF-dependent cancer spread.

3.1353 FcγRI ligation leads to a complex with BLT1 in lipid rafts that enhances rat lung macrophage antimicrobial functions
Serezani, C.H., Aronoff, D.M., Sitrin, R.G. and Peters-Golden, M.
*Blood, **114(15)**, 3316-3324 (2009)*

Leukotriene (LT) B_4 is generated in response to engagement of the Fcγ receptor (FcγR) and potently contributes to FcγR-mediated antimicrobial functions in pulmonary alveolar macrophages. In this study, we report that the LTB_4 receptor leukotriene B_4 receptor 1 (BLT1) redistributes from nonlipid raft (LR) to LR membrane microdomains upon immunoglobulin G–red blood cell, but not LTB_4, challenge. Cholesterol depletion to disrupt LRs abolished LTB_4-induced enhancement of phagocytosis, microbicidal activity, and signaling. The dependence on LR integrity for BLT1 signaling correlated with formation of a complex consisting of BLT1, its primary coupled G protein Gαi3, Src kinase, and FcγRI within LRs. This association was dependent on Src-mediated phosphorylation of BLT1. These data identify a novel form of regulation in which engagement of a macrophage immunoreceptor recruits a stimulatory G protein–coupled receptor into a LR microdomain with resultant enhanced antimicrobial signaling.

3.1354 Ubiquilin and p97/VCP bind erasin, forming a complex involved in ERAD
Lim, P., Danner, R., Liang, J., Doong, H., harman, C., Srinivasan, D., Rothenberg, C., Wang, H., Ye, Y., Fang, S. And Monteiro, M.J.
*J. Cell Biol., **187(2)**, 201-217 (2009)*

Unwanted proteins in the endoplasmic reticulum (ER) are exported into the cytoplasm and degraded by the proteasome through the ER-associated protein degradation pathway (ERAD). Disturbances in ERAD are linked to ER stress, which has been implicated in the pathogenesis of several human diseases. However, the composition and organization of ERAD complexes in human cells is still poorly understood. In this paper, we describe a trimeric complex that we propose functions in ERAD. Knockdown of erasin, a platform for p97/VCP and ubiquilin binding, or knockdown of ubiquilin in human cells slowed degradation of two classical ERAD substrates. In *Caenorhabditis elegans*, ubiquilin and erasin are ER stress-response genes that are regulated by the *ire-1* branch of the unfolded protein response pathway. Loss of ubiquilin or erasin resulted in activation of ER stress, increased accumulation of polyubiquitinated proteins, and shortened lifespan in worms. Our results strongly support a role for this complex in ERAD and in the regulation of ER stress.

3.1355 Filamin A Regulates Caveolae Internalization and Trafficking in Endothelial Cells
Sverdlov, M., Shinin, V., Place, A.T., Castellon, M. and Minshall, R.D.
*Mol. Biol. Cell, **20**, 4531-4540 (2009)*

Transcytosis via caveolae is critical for maintaining vascular homeostasis by regulating the tissue delivery of macromolecules, hormones, and lipids. In the present study, we test the hypothesis that interactions between F-actin cross-linking protein filamin A and caveolin-1 facilitate the internalization and trafficking of caveolae. Small interfering RNA-mediated knockdown of filamin A, but not filamin B, reduced the uptake and transcytosis of albumin by ~35 and 60%, respectively, without altering the actin cytoskeletal structure or cell–cell adherens junctions. Mobility of both intracellular caveolin-1–green fluorescent protein (GFP)-labeled vesicles measured by fluorescence recovery after photobleaching and membrane-associated vesicles measured by total internal reflection-fluorescence microscopy was decreased in cells with reduced filamin A expression. In addition, in melanoma cells that lack filamin A (M2 cells), the majority of caveolin-1-GFP was localized on the plasma membrane, whereas in cells in which filamin A expression

was reconstituted (A7 cells and M2 cells transfected with filamin A-RFP), caveolin-1-GFP was concentrated in intracellular vesicles. Filamin A association with caveolin-1 in endothelial cells was confirmed by cofractionation of these proteins in density gradients, as well as by coimmunoprecipitation. Moreover, this interaction was enhanced by Src activation, associated with increased caveolin-1 phosphorylation, and blocked by Src inhibition. Taken together, these data suggest that filamin A association with caveolin-1 promotes caveolae-mediated transport by regulating vesicle internalization, clustering, and trafficking.

3.1356 Characteristics of alpha/beta interferon induction after infection of murine fibroblasts with wild-type and mutant alphaviruses
Burke, C.W., Gardner, C.L., Steffan, J.J., Ryman, K.D. and Klimstra, W.B.
*Virology, **395**, 121-132 (2009)*

We examined the characteristics of interferon alpha/beta (IFN-α/β) induction after alphavirus or control Sendai virus (SeV) infection of murine fibroblasts (MEFs). As expected, SeV infection of wild-type (wt) MEFs resulted in strong dimerization of IRF3 and the production of high levels of IFN-α/β. In contrast, infection of MEFs with multiple alphaviruses failed to elicit detectable IFN-α/β. In more detailed studies, Sindbis virus (SINV) infection caused dimerization and nuclear migration of IRF3, but minimal IFN-β promoter activity, although surprisingly, the infected cells were competent for IFN production by other stimuli early after infection. A SINV mutant defective in host macromolecular synthesis shutoff induced IFN-α/β in the MEF cultures dependent upon the activities of the TBK1 IRF3 activating kinase and host pattern recognition receptors (PRRs) PKR and MDA5 but not RIG-I. These results suggest that wild-type alphaviruses antagonize IFN induction after IRF3 activation but also may avoid detection by host PRRs early after infection.

3.1357 N-Myristoylation targets dihydroceramide Δ4-desaturase 1 to mitochondria: Partial involvement in the apoptotic effect of myristic acid
Beauchamp, E., Tekpli, X., Marteil, g., Lagadic-Gossmann, D., Legrand, P. And Rioux, V.
*Biochimie, **91**, 1411-1419 (2009)*

This study was designed to analyze the effect of myristic acid on ceramide synthesis and its related lipoapoptosis pathway. It was previously observed that myristic acid binds dihydroceramide Δ4-desaturase 1 (DES1) through N-myristoylation and activates this enzyme involved in the final de novo ceramide biosynthesis step. In the present study, we show first by immunofluorescence microscopy and subcellular fractionation that DES1 myristoylation targets part of the recombinant protein to the mitochondria in COS-7 cells. In addition, native dihydroceramide Δ4-desaturase activity was found in both the endoplasmic reticulum and mitochondria in rat hepatocytes. Dihydroceramide conversion to ceramide was increased in COS-7 cells expressing DES1 and incubated with myristic acid. The expression of the wild-type myristoylable DES1-Gly alone, but not the expression of the unmyristoylable mutant DES1-Ala, induced apoptosis of COS-7 cells. Finally, myristic acid alone also increased the production of cellular ceramide and had an apoptotic effect. This effect was potentiated on caspase activity when the myristoylable form of DES1 was expressed. Therefore, these results suggest that the myristoylation of DES1 can target the enzyme to the mitochondria leading to an increase in ceramide levels which in turn contributes to partially explain the apoptosis effect of myristic acid in COS-7 cells.

3.1358 Outer Membrane Machinery and Alginate Synthesis Regulators Control Membrane Vesicle Production in Pseudomonas aeruginosa
Tashiro, Y., Sakai, R., Toyofuku, M., Sawada, I., Nakajima-Kambe, T., Uchiyama, H. and Nomura, N.
*J. Bacteriol., **191(24)**, 7509-7519 (2009)*

The opportunistic human bacterial pathogen *Pseudomonas aeruginosa* produces membrane vesicles (MVs) in its surrounding environment. Several features of the *P. aeruginosa* MV production mechanism are still unknown. We previously observed that depletion of Opr86, which has a role in outer membrane protein (OMP) assembly, resulted in hypervesiculation. In this study, we showed that the outer membrane machinery and alginate synthesis regulatory machinery are closely related to MV production in *P. aeruginosa*. Depletion of Opr86 resulted in increased expression of the periplasmic serine protease MucD, suggesting that the accumulation of misfolded OMPs in the periplasm is related to MV production. Indeed, the *mucD* mutant showed a mucoid phenotype and the *mucD* mutation caused increased MV production. Strains with the gene encoding alginate synthetic regulator AlgU, MucA, or MucB deleted also caused altered MV production. Overexpression of either MucD or AlgW serine proteases resulted in decreased

MV production, suggesting that proteases localized in the periplasm repress MV production in *P. aeruginosa*. Deletion of *mucD* resulted in increased MV proteins, even in strains with mutations in the *Pseudomonas* quinolone signal (PQS), which serves as a positive regulator of MV production. This study suggests that misfolded OMPs may be important for MV production, in addition to PQS, and that these regulators act in independent pathways.

3.1359 Bovine viral diarrhea virus NS4B protein is an integral membrane protein associated with Golgi markers and rearranged host membranes

Weiskircher, E., Aligo, J., Ning, G. and Konan, K.V.
Virol. J., ***6***, *185-199 (2009)*

Background
Very little is known about BVDV NS4B, a protein of approximately 38 kDa. However, a missense mutation in NS4B has been implicated in changing BVDV from a cytopathic to noncytopathic virus, suggesting that NS4B might play a role in BVDV pathogenesis. Though this is one possible function, it is also likely that NS4B plays a role in BVDV genome replication. For example, BVDV NS4B interacts with NS3 and NS5A, implying that NS4B is part of a complex, which contains BVDV replicase proteins. Other possible BVDV NS4B functions can be inferred by analogy to hepatitis C virus (HCV) NS4B protein. For instance, HCV NS4B remodels host membranes to form the so-called membranous web, the site for HCV genome replication. Finally, HCV NS4B is membrane-associated, implying that HCV NS4B may anchor the virus replication complex to the membranous web structure. Unlike its HCV counterpart, we know little about the subcellular distribution of BVDV NS4B protein. Further, it is not clear whether NS4B is localized to host membrane alterations associated with BVDV infection.
Results
We show first that release of infectious BVDV correlates with the kinetics of BVDV genome replication in infected cells. Secondly, we found that NS4B subcellular distribution changes over the course of BVDV infection. Further, BVDV NS4B is an integral membrane protein, which colocalizes mainly with the Golgi compartment when expressed alone or in the context of BVDV infection. Additionally, BVDV induces host membrane rearrangement and these membranes contain BVDV NS4B protein. Finally, NS4B colocalizes with replicase proteins NS5A and NS5B proteins, raising the possibility that NS4B is a component of the BVDV replication complex. Interestingly, NS4B was found to colocalize with mitochondria suggesting that this organelle might play a role in BVDV genome replication or cytopathogenicity.
Conclusion
These results show that BVDV NS4B is an integral membrane protein associated with the Golgi apparatus and virus-induced membranes, the putative site for BVDV genome replication. On the basis of NS4B Colocalization with NS5A and NS5B, we conclude that NS4B protein is an integral component of the BVDV replication complex.

3.1360 EFFECT OF SIMVASTATIN ON GLIOMA CELL PROLIFERATION, MIGRATION, AND APOPTOSIS

Wu, H., Jiang, H., Lu, D., Xiong, Y., Qu, C., Zhou, D., Mahmood, A. and Chopp, M.
Neurosurgery, ***65(6)***, *1087-1097 (2009)*

OBJECTIVE: In this study, we investigated the effects of simvastatin on proliferation, migration, and apoptosis in human U251 and U87 glioma cells and the underlying molecular mechanism.
METHODS: We used colony formation assay to test the cell proliferation, in vitro scratch assay to examine the cell migration, and caspase-3 activity assay, annexin V staining, and cytochrome C release to evaluate the cell apoptosis. Lipid raft fractions were isolated from glioma cells. Total cholesterol content assay was used to test the change of cholesterol level in lipid raft fractions. Immunocytochemistry staining was performed to detect the changes of lipid rafts in cell membranes. Western blotting analysis was performed to examine the signal transduction both in cells and in lipid raft fractions.
RESULTS: Simvastatin inhibited proliferation and migration of U251 and U87 cells dose dependently. Simvastatin induced an increase of caspase-3 activity and annexin V staining, and down-regulated the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Simvastatin also decreased cholesterol content in lipid raft fractions, suppressed caveolin-1 expression in the lipid rafts, and induced Fas translocation into lipid rafts, suggesting that simvastatin may inhibit the prosurvival PI3K/Akt pathway and trigger caspase-3-dependent apoptotic cell death through the modulation of lipid rafts.

CONCLUSION: These results suggest that modulation of lipid rafts, Fas translocation, and PI3K/Akt/caspase-3 pathway are involved in the antitumor effect of simvastatin and may have a potential role in cancer prevention and treatment.

3.1361 APP Anterograde Transport Requires Rab3A GTPase Activity for Assembly of the Transport Vesicle

Szodorai, A., Kuan, Y-H., Hunzelmann, S., Engel, U., Sakane, A., Sasaki, T., Takai, Y., Kirsch, J., Müller, U., Beyreuther, K., Brady, S., Morfini, G. and Kins, S.
J. Neurosci., ***29(46)***, *14534-14544 (2009)*

The amyloid precursor protein (APP) is anterogradely transported by conventional kinesin in a distinct transport vesicle, but both the biochemical composition of such a vesicle and the specific kinesin-1 motor responsible for transport are poorly defined. APP may be sequentially cleaved by β- and γ-secretases leading to accumulation of β-amyloid (Aβ) peptides in brains of Alzheimer's disease patients, whereas cleavage of APP by α-secretases prevents Aβ generation. Here, we demonstrate by time-lapse analysis and immunoisolations that APP is a cargo of a vesicle containing the kinesin heavy chain isoform kinesin-1C, the small GTPase Rab3A, and a specific subset of presynaptic protein components. Moreover, we report that assembly of kinesin-1C and APP in this vesicle type requires Rab3A GTPase activity. Finally, we show cleavage of APP in transport vesicles by α-secretase activity, likely mediated by ADAM10. Together, these data indicate that maturation of APP transport vesicles, including recruitment of conventional kinesin, requires Rab3 GTPase activity.

3.1362 Cyclodextrin overcomes deficient lysosome-to-endoplasmic reticulum transport of cholesterol in Niemann-Pick type C cells

Abi-Mosleh, L., Infante, R.E., Radhakrishnan, A., Goldstein, J.L. and Brown, M.S.
PNAS, ***106(46)***, *19316-19321 (2009)*

A handoff model has been proposed to explain the egress from lysosomes of cholesterol derived from receptor-mediated endocytosis of LDL. Cholesterol is first bound by soluble Niemann-Pick C2 (NPC2) protein, which hands off the cholesterol to the N-terminal domain of membrane-bound NPC1. Cells lacking NPC1 or NPC2 accumulate LDL-derived cholesterol in lysosomes and fail to deliver LDL cholesterol to the endoplasmic reticulum (ER) for esterification by acyl-CoA acyltransferase (ACAT) and for inhibition of sterol regulatory element-binding protein cleavage. Here, we support this model by showing that the cholesterol transport defect in NPC1 mutant cells is restricted to lysosomal export. Other cholesterol transport pathways appear normal, including the movement of cholesterol from the plasma membrane to the ER after treatment of cells with 25-hydroxycholesterol or sphingomyelinase. The NPC1 or NPC2 block in cholesterol delivery to the ER can be overcome by 2-hydroxypropyl-β-cyclodextrin, which leads to a marked increase in ACAT-mediated cholesterol esterification. The buildup of cholesteryl esters in the cytosol is expected to be much less toxic than the buildup of free cholesterol in the lysosomes of patients with mutations in NPC1 or NPC2.

3.1363 Immobilization of the Glycosylphosphatidylinositol-anchored Gas1 Protein into the Chitin Ring and Septum Is Required for Proper Morphogenesis in Yeast

Rolli, E., Ragni, E., calderon, J., Porello, S., Fascio, U. and Popolo, L.
Mol. Biol. Cell, ***20***, *4856-4870 (2009)*

Gas1p is a glucan-elongase that plays a crucial role in yeast morphogenesis. It is predominantly anchored to the plasma membrane through a glycosylphosphatidylinositol, but a fraction was also found covalently bound to the cell wall. We have used fusions with the green fluorescent protein **or** red fluorescent protein (RFP) to determine its localization. Gas1p was present in microdomains of the plasma membrane, at the mother-bud neck and in the bud scars. By exploiting the instability of RFP-Gas1p, we identified mobile and immobile pools of Gas1p. Moreover, in *chs3*Δ cells the chitin ring and the cross-linked Gas1p were missing, but this unveiled an additional unexpected localization of Gas1p along the septum line in cells at cytokinesis. Localization of Gas1p was also perturbed in a *chs2*Δ mutant where a remedial septum is produced. Phenotypic analysis of cells expressing a fusion of Gas1p to a transmembrane domain unmasked new roles of the cell wall-bound Gas1p in the maintenance of the bud neck size and in cell separation. We present evidence that Crh1p and Crh2p are required for tethering Gas1p to the chitin ring and bud scar. These results reveal a new mechanism of protein immobilization at specific sites of the cell envelope.

3.1364 **Inhibition of acyl-coenzyme A: cholesterol acyl transferase modulates amyloid precursor protein trafficking in the early secretory pathway**
Huttunen, H.J., Peach, C., Bhattacharyya, R., Barren, C., Pettingell, W., Hutter-Paier, B., Windisch, M., Berezovska, O. and Kovacs, D.M.
FASEB J., ***23***, *3819-3828 (2009)*

Amyloid β-peptide (Aβ) has a central role in the pathogenesis of Alzheimer's disease (AD). Cellular cholesterol homeostasis regulates endoproteolytic generation of Aβ from the amyloid precursor protein (APP). Previous studies have identified acyl-coenzyme A: cholesterol acyltransferase (ACAT), an enzyme that regulates subcellular cholesterol distribution, as a potential therapeutic target for AD. Inhibition of ACAT activity decreases Aβ generation in cell- and animal-based models of AD through an unknown mechanism. Here we show that ACAT inhibition retains a fraction of APP molecules in the early secretory pathway, limiting the availability of APP for secretase-mediated proteolytic processing. ACAT inhibitors delayed the trafficking of immature APP molecules from the endoplasmic reticulum (ER) as shown by metabolic labeling and live-cell imaging. This resulted in partial ER retention of APP and enhanced ER-associated degradation of APP by the proteasome, without activation of the unfolded protein response pathway. The ratio of mature APP to immature APP was reduced in brains of mice treated with ACAT inhibitors, and strongly correlated with reduced brain APP-C99 and cerebrospinal fluid Aβ levels in individual animals. Our results identify a novel ACAT-dependent mechanism that regulates secretory trafficking of APP, likely contributing to decreased Aβ generation *in vivo*.—Huttunen, H. J., Peach, C., Bhattacharyya, R., Barren, C., Pettingell, W., Hutter-Paier, B., Windisch, M., Berezovska, O., Kovacs, D. M. Inhibition of acyl-coenzyme A: cholesterol acyl transferase modulates amyloid precursor protein trafficking in the early secretory pathway.

3.1365 **Peroxisomal proteomics: Biomonitoring in mussels after the *Prestige*'s oil spill**
Apraiz, I., Cajaraville, M.P. and Cristobal, S.
Marine Pollution Bulletin, ***58***, *1815-1826 (2009)*

Peroxisomal proteomics was applied to assess possible biological effects after the *Prestige*'s oil spill. Mussels were sampled in July 2004 and 2005 in four stations in the NW (closest to the spill) and NE coasts of the Iberian Peninsula. Principal components analysis (PCA) suggested differences in protein expression among stations and sampling years. Several proteins were putatively identified by mass spectrometry and immunolocalization. PC1 separated the NW stations in 2004 from the rest of the stations and sampling years mainly due to up-regulation of peroxisomal β-oxidation proteins and PMP70. PC3 separated the NE stations, based on up-regulation of the antioxidant enzyme catalase in 2004 compared to 2005. PC4 separated the stations in the NE and the NW. This work shows that environmental proteomics, together with multivariate data analysis, could provide information to interpret the effects of oil spills at cellular level in mussels.

3.1366 **Palmitoylation of Hepatitis C Virus Core Protein Is Important for Virion Production**
Majeau, N., Fromentin, R., Savard, C., Duval, M., Tremblay, M.J. and Leclerc, D.
J. Biol. Chem., ***284(49)***, *33915-33925 (2009)*

Hepatitis C virus core protein is the viral nucleocapsid of hepatitis C virus. Interaction of core with cellular membranes like endoplasmic reticulum (ER) and lipid droplets (LD) appears to be involved in viral assembly. However, how these interactions with different cellular membranes are regulated is not well understood. In this study, we investigated how palmitoylation, a post-translational protein modification, can modulate the targeting of core to cellular membranes. We show that core is palmitoylated at cysteine 172, which is adjacent to the transmembrane domain at the C-terminal end of core. Site-specific mutagenesis of residue Cys^{172} showed that palmitoylation is not involved in the maturation process carried out by the signal peptide peptidase or in the targeting of core to LD. However, palmitoylation was shown to be important for core association with smooth ER membranes and ER closely surrounding LDs. Finally, we demonstrate that mutation of residue Cys^{172} in the J6/JFH1 virus genome clearly impairs virion production.

3.1367 **Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells**
Hong, B.S., Cho, J-H., Kim, H., Choi, E-J., Rho, S., Kim, J., Kim, J.H., Choi, D-S., Kim, Y-K., Hwang, D. and Gho, Y.S.
BMC Genomics, ***10***, *556-568 (2009)*

Background
Various cancer cells, including those of colorectal cancer (CRC), release microvesicles (exosomes) into surrounding tissues and peripheral circulation. These microvesicles can mediate communication between cells and affect various tumor-related processes in their target cells.
Results
We present potential roles of CRC cell-derived microvesicles in tumor progression via a global comparative microvesicular and cellular transcriptomic analysis of human SW480 CRC cells. We first identified 11,327 microvesicular mRNAs involved in tumorigenesis-related processes that reflect the physiology of donor CRC cells. We then found 241 mRNAs enriched in the microvesicles above donor cell levels, of which 27 were involved in cell cycle-related processes. Network analysis revealed that most of the cell cycle-related microvesicle-enriched mRNAs were associated with M-phase activities. The integration of two mRNA datasets showed that these M-phase-related mRNAs were differentially regulated across CRC patients, suggesting their potential roles in tumor progression. Finally, we experimentally verified the network-driven hypothesis by showing a significant increase in proliferation of endothelial cells treated with the microvesicles.
Conclusion
Our study demonstrates that CRC cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells, suggesting that microvesicles of cancer cells can be involved in tumor growth and metastasis by facilitating angiogenesis-related processes. This information will help elucidate the pathophysiological functions of tumor-derived microvesicles, and aid in the development of cancer diagnostics, including colorectal cancer.

3.1368 Cystin Localizes to Primary Cilia via Membrane Microdomains and a Targeting Motif

Tao, B., bu, S., Yang, Z., Siroky, B., Kappes, J.C., Kispert, A. and Guay-Woodford, L.M.

J. Am. Soc. Nephrol., ***20***, *2570-2580 (2009)*

Primary cilia are dynamic, complex structures that contain >500 proteins, including several related to polycystic kidney disease. How these proteins target to cilia and assemble is unknown. We previously identified *Cys1* as the gene responsible for disease in *Cys1*^{cpk} mice, a mouse model of autosomal recessive polycystic kidney disease; this gene encodes cystin, a 145–amino acid cilium-associated protein. Here, we characterized the localization of cystin in the embryonic kidney and liver, in isolated renal collecting ducts, and in an inner medullary collecting duct mouse cell line. Because endogenous levels of cystin expression are low, we generated inner medullary collecting duct cell lines that stably express enhanced green fluorescence protein–tagged constructs of wild-type cystin or various truncation mutants. We determined that cystin is myristoylated at its G2 residue and that N-myristoylated cystin fractionates with membrane microdomains. Furthermore, the N-myristoylation signal is necessary but not sufficient to target cystin to the primary cilium. Analysis of deletion and chimeric constructs identified an AxEGG motif that is necessary to target and retain cystin in the cilium. Derangement of these localization motifs may lead to cystic kidney disease.

3.1369 Oral PEG 15–20 protects the intestine against radiation: role of lipid rafts

Valuckaite, V., Zaborina, O., Long, J., Hauer-Jensen, M., Wang, J., Holbrook, C., Zaborin, A., Drabik, K., Katdare, M., Mauceri, H., Weichselbaum, R., Firestone, M.A., Lee, K.Y., Chang, E.B., Matthews, J. and Alverdy, J.C.

Am. J. Physiool. Gastrointest. Liver Physiol., ***297***, *G1041-G1052 (2009)*

Intestinal injury following abdominal radiation therapy or accidental exposure remains a significant clinical problem that can result in varying degrees of mucosal destruction such as ulceration, vascular sclerosis, intestinal wall fibrosis, loss of barrier function, and even lethal gut-derived sepsis. We determined the ability of a high-molecular-weight polyethylene glycol-based copolymer, PEG 15–20, to protect the intestine against the early and late effects of radiation in mice and rats and to determine its mechanism of action by examining cultured rat intestinal epithelia. Rats were exposed to fractionated radiation in an established model of intestinal injury, whereby an intestinal segment is surgically placed into the scrotum and radiated daily. Radiation injury score was decreased in a dose-dependent manner in rats gavaged with 0.5 or 2.0 g/kg per day of PEG 15–20 (n = 9–13/group, $P < 0.005$). Complementary studies were performed in a novel mouse model of abdominal radiation followed by intestinal inoculation with *Pseudomonas aeruginosa* (*P. aeruginosa*), a common pathogen that causes lethal gut-derived sepsis following radiation. Mice mortality was decreased by 40% in mice drinking 1% PEG 15–20 (n = 10/group, $P < 0.001$). Parallel studies were performed in cultured rat intestinal epithelial cells treated with PEG 15–

20 before radiation. Results demonstrated that PEG 15–20 prevented radiation-induced intestinal injury in rats, prevented apoptosis and lethal sepsis attributable to *P. aeruginosa* in mice, and protected cultured intestinal epithelial cells from apoptosis and microbial adherence and possible invasion. PEG 15–20 appeared to exert its protective effect via its binding to lipid rafts by preventing their coalescence, a hallmark feature in intestinal epithelial cells exposed to radiation.

3.1370 Neural Cell Adhesion Molecule Modulates Dopaminergic Signaling and Behavior by Regulating Dopamine D2 Receptor Internalization
Xiao, M-F., Xu, J-C., Tereshchenko, Y., Novak, D., Schachner, M. and Kleene, R.
J. Neurosci., ***29(47)***, *14752-14763 (2009)*

The dopaminergic system plays an important role in the etiology of schizophrenia, and most antipsychotic drugs exert their functions by blocking dopamine D_2 receptors (D_2Rs). Since the signaling strength mediated by D_2Rs is regulated by internalization and degradation processes, it is crucial to identify molecules that modulate D_2R localization at the cell surface. Here, we show that the neural cell adhesion molecule (NCAM) promotes D_2R internalization/desensitization and subsequent degradation via direct interaction with a short peptide in the third intracellular loop of the D_2R. NCAM deficiency in mice leads to increased numbers of D_2Rs at the cell surface and augmented D_2R signaling as a result of impaired D_2R internalization. Furthermore, NCAM-deficient mice show higher sensitivity to the psychostimulant apomorphine and exaggerated activity of dopamine-related locomotor behavior. These results demonstrate that, in addition to its classical function in cell adhesion, NCAM is involved in regulating the trafficking of the neurotransmitter receptor D_2R as well as receptor-mediated signaling and behavior, thus implicating NCAM as modulator of the dopaminergic system and a potential pharmacological target for dopamine-related neurological and psychiatric disorders.

3.1371 M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex
Hase, K., Kimura, S., Takatsu, H., Ohmae, M., Kawano, S., Kitamura, H., Ito, M., Waterai, H., Clayton Hazelett, C., Yeaman, C. and Ohno, H.
Nature Cell Biol., ***11(12)***, *1427-1432 (2009)*

Cell–cell communication is essential for the development and homeostasis of multicellular organisms. Recently, a new type of cell–cell communication was discovered that is based on the formation of thin membranous nanotubes between remote cells[1, 2]. These long membrane tethers, termed tunneling nanotubes (TNTs), form an intercellular conduit and have been shown to enable the transport of various cellular components and signals. However, the molecular basis for TNT formation remains to be elucidated. Here we report that a mammalian protein, M-Sec, induces *de novo* formation of numerous membrane protrusions extending from the plasma membrane, some of which tether onto adjacent cells and subsequently form TNT-like structures. Depletion of M-Sec by RNA interference (RNAi) greatly reduced endogenous TNT formation as well as intercellular propagation of a calcium flux in a macrophage cell line. Furthermore, blockage of the interaction of M-Sec with Ral and the exocyst complex, which serves as a downstream effector of Ral, attenuated the formation of membrane nanotubes. Our results reveal that M-Sec functions as a key regulator of membrane nanotube formation through interaction with the Ral–exocyst pathway.

3.1372 Identification of novel proteins in isolated polyphosphate vacuoles in the primitive red alga Cyanidioschyzon merolae
Yagisawa, F., Nishida, K., Yoshida, M., Ohnuma, M., Shimada, T., Fujiwara, T., Yoshida, Y., Misumi, O., Kuroiwa, H. and Kuroiwa, T.
Plant. J., ***60***, *882-893 (2009)*

Plant vacuoles are organelles bound by a single membrane, and involved in various functions such as intracellular digestion, metabolite storage, and secretion. To understand their evolution and fundamental mechanisms, characterization of vacuoles in primitive plants would be invaluable. Algal cells often contain polyphosphate-rich compartments, which are thought to be the counterparts of seed plant vacuoles. Here, we developed a method for isolating these vacuoles from Cyanidioschyzon merolae, and identified their proteins by MALDI TOF-MS. The vacuoles were of unexpectedly high density, and were highly enriched at the boundary between 62 and 80% w/v iodixanol by density-gradient ultracentrifugation. The vacuole-containing fraction was subjected to SDS-PAGE, and a total of 46 proteins were identified, including six lytic enzymes, 13 transporters, six proteins for membrane fusion or vesicle trafficking, five non-lytic enzymes, 13 proteins of unknown function, and three miscellaneous proteins. Fourteen proteins were

homologous to known vacuolar or lysosomal proteins from seed plants, yeasts or mammals, suggesting functional and evolutionary relationships between C. merolae vacuoles and these compartments. The vacuolar localization of four novel proteins, namely CMP249C (metallopeptidase), CMJ260C (prenylated Rab receptor), CMS401C (ABC transporter) and CMT369C (o-methyltransferase), was confirmed by labeling with specific antibodies or transient expression of hemagglutinin-tagged proteins. The results presented here provide insights into the proteome of C. merolae vacuoles and shed light on their functions, as well as indicating new features.

3.1373 Stomatin-like Protein-1 Interacts with Stomatin and Is Targeted to Late Endosomes
Mairhofer, M., Steiner, M., Salzer, U. and Prohaska, R.
J. Biol. Chem., ***284(42)***, *29218-29229 (2009)*

The human stomatin-like protein-1 (SLP-1) is a membrane protein with a characteristic bipartite structure containing a stomatin domain and a sterol carrier protein-2 (SCP-2) domain. This structure suggests a role for SLP-1 in sterol/lipid transfer and transport. Because SLP-1 has not been investigated, we first studied the molecular and cell biological characteristics of the expressed protein. We show here that SLP-1 localizes to the late endosomal compartment, like stomatin. Unlike stomatin, SLP-1 does not localize to the plasma membrane. Overexpression of SLP-1 leads to the redistribution of stomatin from the plasma membrane to late endosomes suggesting a complex formation between these proteins. We found that the targeting of SLP-1 to late endosomes is caused by a GY*XX*Φ (Φ being a bulky, hydrophobic amino acid) sorting signal at the N terminus. Mutation of this signal results in plasma membrane localization. SLP-1 and stomatin co-localize in the late endosomal compartment, they co-immunoprecipitate, thus showing a direct interaction, and they associate with detergent-resistant membranes. In accordance with the proposed lipid transfer function, we show that, under conditions of blocked cholesterol efflux from late endosomes, SLP-1 induces the formation of enlarged, cholesterol-filled, weakly LAMP-2-positive, acidic vesicles in the perinuclear region. This massive cholesterol accumulation clearly depends on the SCP-2 domain of SLP-1, suggesting a role for this domain in cholesterol transfer to late endosomes.

3.1374 Ceramide kinase regulates phospholipase C and phosphatidylinositol 4, 5, bisphosphate in phototransduction
Dasgupta, U., Bamba, T., Chiantia, S., Karim, P., Tayoun, A.N.A., Yonamine, I., Rawat, S.S., Rao, R.P., Nagashima, K., Fukusaki, E., Puri, V., Dolph, P.J., Schwille, P., Acharya, J.K. and Acharya, U.
PNAS, ***106(47)***, *20063-20068 (2009)*
Phosphoinositide-specific phospholipase C (PLC) is a central effector for many biological responses regulated by G-protein–coupled receptors including Drosophila phototransduction where light sensitive channels are activated downstream of NORPA, a PLCβ homolog. Here we show that the sphingolipid biosynthetic enzyme, ceramide kinase, is a novel regulator of PLC signaling and photoreceptor homeostasis. A mutation in ceramide kinase specifically leads to proteolysis of NORPA, consequent loss of PLC activity, and failure in light signal transduction. The mutant photoreceptors also undergo activity-dependent degeneration. Furthermore, we show that a significant increase in ceramide, resulting from lack of ceramide kinase, perturbs the membrane microenvironment of phosphatidylinositol 4, 5, bisphosphate (PIP_2), altering its distribution. Fluorescence image correlation spectroscopic studies on model membranes suggest that an increase in ceramide decreases clustering of PIP_2 and its partitioning into ordered membrane domains. Thus ceramide kinase–mediated maintenance of ceramide level is important for the local regulation of PIP_2 and PLC during phototransduction.

3.1375 The amino terminus of tau inhibits kinesin-dependent axonal transport: Implications for filament toxicity
LaPointe, N.E., Morfini, G., Pigino, G., Gaisina, I.N., Kozikowski, A.P., Binder, L.I. and Brady, S.T.
J. Neurosic. Res., ***87(2)***, *440-451 (2009)*

The neuropathology of Alzheimer's disease (AD) and other tauopathies is characterized by filamentous deposits of the microtubule-associated protein tau, but the relationship between tau polymerization and neurotoxicity is unknown. Here, we examined effects of filamentous tau on fast axonal transport (FAT) using isolated squid axoplasm. Monomeric and filamentous forms of recombinant human tau were perfused in axoplasm, and their effects on kinesin- and dynein-dependent FAT rates were evaluated by video microscopy. Although perfusion of monomeric tau at physiological concentrations showed no effect, tau filaments at the same concentrations selectively inhibited anterograde (kinesin-dependent) FAT, triggering the release of conventional kinesin from axoplasmic vesicles. Pharmacological experiments indicated that the effect of tau filaments on FAT is mediated by protein phosphatase 1 (PP1) and glycogen

synthase kinase-3 (GSK-3) activities. Moreover, deletion analysis suggested that these effects depend on a conserved 18-amino-acid sequence at the amino terminus of tau. Interestingly, monomeric tau isoforms lacking the C-terminal half of the molecule (including the microtubule binding region) recapitulated the effects of full-length filamentous tau. Our results suggest that pathological tau aggregation contributes to neurodegeneration by altering a regulatory pathway for FAT.

3.1376 Content of endoplasmic reticulum and Golgi complex membranes positively correlates with the proliferative status of brain cells

Silvestre, D.C., Maccioni, H.J.F. and Caputto, B.L.
J. Neurosci. Res., ***87(4)***, *857-865 (2009)*

Although the molecular and cellular basis of particular events that lead to the biogenesis of membranes in eukaryotic cells has been described in detail, understanding of the intrinsic complexity of the pleiotropic response by which a cell adjusts the overall activity of its endomembrane system to accomplish these requirements is limited. Here we carried out an immunocytochemical and biochemical examination of the content and quality of the endoplasmic reticulum (ER) and Golgi apparatus membranes in two in vivo situations characterized by a phase of active cell proliferation followed by a phase of declination in proliferation (rat brain tissue at early and late developmental stages) or by permanent active proliferation (gliomas and their most malignant manifestation, glioblastomas multiforme). It was found that, in highly proliferative phases of brain development (early embryo brain cells), the content of ER and Golgi apparatus membranes, measured as total lipid phosphorous content, is higher than in adult brain cells. In addition, the concentration of protein markers of ER and Golgi is also higher in early embryo brain cells and in human glioblastoma multiforme cells than in adult rat brain or in nonpathological human brain cells. Results suggest that the amount of endomembranes and the concentration of constituent functional proteins diminish as cells decline in their proliferative activity. Although the molecular and cellular basis of particular events that lead to the biogenesis of membranes in eukaryotic cells has been described in detail, understanding of the intrinsic complexity of the pleiotropic response by which a cell adjusts the overall activity of its endomembrane system to accomplish these requirements is limited. Here we carried out an immunocytochemical and biochemical examination of the content and quality of the endoplasmic reticulum (ER) and Golgi apparatus membranes in two in vivo situations characterized by a phase of active cell proliferation followed by a phase of declination in proliferation (rat brain tissue at early and late developmental stages) or by permanent active proliferation (gliomas and their most malignant manifestation, glioblastomas multiforme). It was found that, in highly proliferative phases of brain development (early embryo brain cells), the content of ER and Golgi apparatus membranes, measured as total lipid phosphorous content, is higher than in adult brain cells. In addition, the concentration of protein markers of ER and Golgi is also higher in early embryo brain cells and in human glioblastoma multiforme cells than in adult rat brain or in nonpathological human brain cells. Results suggest that the amount of endomembranes and the concentration of constituent functional proteins diminish as cells decline in their proliferative activity.

3.1377 The two-hydrophobic domain tertiary structure of reticulon proteins is critical for modulation of β-secretase BACE1

Kume, H., Murayama, K.S. and Araki,, W.
J. Neurosci. Res., ***87(13)***, *2963-2972 (2009)*

β-Site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) is a membrane-bound protease that is essential for the production of β-amyloid protein (Aβ). Given the crucial role of Aβ accumulation in Alzheimer's disease (AD), inhibition of BACE1 activity may represent a feasible therapeutic strategy in the treatment of AD. Recently, we and others identified reticulon 3 (RTN3) and reticulon 4-B/C (RTN4-B/C or Nogo-B/C) as membrane proteins that interact with BACE1 and inhibit its ability to produce Aβ. In this study, we employed various mutants of RTN3 and RTN4-C and C. elegans RTN to investigate the molecular mechanisms by which RTNs regulate BACE1. We found that RTN3 mutants lacking the N-terminal or C-terminal or loop domain as well as a RTN4-C mutant lacking the C-terminal domain bound to BACE1 comparably to wild-type RTN3 and RTN4-C. Furthermore, overexpression of wild-type RTN3, RTN4-C, and these RTN mutants similarly reduced Aβ40 and Aβ42 secretion by cells expressing Swedish mutant APP. C. elegans RTN, which has low homology to human RTNs, also interacted with BACE1 and inhibited Aβ secretion. In contrast, two RTN3 mutants containing deletions of the first or second potential transmembrane domains and an RTN3 swap mutant of the second transmembrane domain bound BACE1 but failed to inhibit Aβ secretion. Collectively, these results suggest that the two-transmembrane-domain

tertiary structure of RTN proteins is critical for the ability of RTNs to modulate BACE1 activity, whereas N-terminal, C-terminal and loop regions are not essential for this function.

3.1378 **Proteome of plant peroxisomes: new perspectives on the role of these organelles in cell biology**
Palma, J.M., Corpas, F.J. and del Rio, L.A.
Proteomics, ***9(9)***, *2301-2312 (2009)*

Peroxisomes are cell organelles bounded by a single membrane with a basically oxidative metabolism. Peroxisomes house catalase and H_2O_2-producing flavin-oxidases as the main protein constituents. However, since their discovery in early fifties, a number of new enzymes and metabolic pathways have been reported to be also confined to these organelles. Thus, the presence of exo- and endo-peptidases, superoxide dismutases, the enzymes of the plant ascorbate-glutathione cycle plus ascorbate and glutathione, several NADP-dehydrogenases, and also L-arginine-dependent nitric oxide synthase activity has evidenced the relevant role of these organelles in cell physiology. In recent years, the study of new functions of peroxisomes has become a field of intensive research in cell biology, and these organelles have been proposed to be a source of important signal molecules for different transduction pathways. In plants, peroxisomes participate in seed germination, leaf senescence, fruit maturation, response to abiotic and biotic stress, photomorphogenesis, biosynthesis of the plant hormones jasmonic acid and auxin, and in cell signaling by reactive oxygen and nitrogen species (ROS and RNS, respectively). In order to decipher the nature and specific role of the peroxisomal proteins in these processes, several approaches including *in vivo* and *in vitro* import assays and generation of mutants have been used. In the last decade, the development of genomics and the report of the first plant genomes provided plant biologists a powerful tool to assign to peroxisomes those proteins which harbored any of the two peroxisomal targeting signals (PTS, either PTS1 or PTS2) described so far. Unfortunately, those molecular approaches could not give any response to those proteins previously localized in plant peroxisomes by classical biochemical and cell biology methods that did not contain any PTS. However, more recently, proteomic studies of highly purified organelles have provided evidence of the presence in peroxisomes of new proteins not previously reported. Thus, the contribution of proteomic approaches to the biology of peroxisomes is essential, not only for elucidation of the mechanisms involved in the import of the PTS1- and PTS2-independent proteins, but also to the understanding of the role of these organelles in the cell physiology of plant growth and development.

3.1379 **Proteomic identification of proteins translocated to membrane microdomains upon treatment of fibroblasts with the glycosphingolipid, C8-β-D-lactosylceramide**
Kim, S-y., Wang, T-k., Singh, R.D., Wheatley, C.L., Marks, D.L. and Pagano, R.E.
Proteomics, ***9(18)***, *4321-4328 (2009)*

Plasma membrane (PM) microdomains, including caveolae and other cholesterol-enriched subcompartments, are involved in the regulation of many cellular processes, including endocytosis, attachment and signaling. We recently reported that brief incubation of human skin fibroblasts with the synthetic glycosphingolipid, D-*erythro*-octanoyl-lactosylceramide (C8-D-*e*-LacCer), stimulates endocytosis *via* caveolae and induces the appearance of micron-size microdomains on the PM. To further understand the effects of C8-D-*e*-LacCer treatment on PM microdomains, we used a detergent-free method to isolate microdomain-enriched membranes from fibroblasts treated ±C8-D-*e*-LacCer, and performed 2-DE and mass spectrophotometry to identify proteins that were altered in their distribution in microdomains. Several proteins were identified in the microdomain-enriched fractions, including lipid transfer proteins and proteins related to the functions of small GTPases. One protein, Rho-associated protein kinase 2 (ROCK2), was verified by Western blotting to occur in microdomain fractions and to increase in these fractions after D-*e*-LacCer treatment. Immunofluorescence revealed that ROCK2 exhibited an increased localization at or near the PM in C8-D-*e*-LacCer-treated cells. In contrast, ROCK2 distribution in microdomains was decreased by treatment of cells with C8-L-*threo*-lactosylceramide, a glycosphingolipid with non-natural stereochemistry. This study identifies new microdomain-associated proteins and provides evidence that microdomains play a role in the regulation of the Rho/ROCK signaling pathway.

3.1380 **Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles**
Lee, E-Y., Choi, D-Y., Kim, D-K., Kim, J-W., Park, J O., Kim, S., Kim, S-H., Desiderio, D.M., Kim, Y-K., Kim, K-P- and Gho, Y.S.
Proteomics, ***9(24)***, *5425-5436 (2009)*

Although archaea, Gram-negative bacteria, and mammalian cells constitutively secrete membrane vesicles (MVs) as a mechanism for cell-free intercellular communication, this cellular process has been overlooked in Gram-positive bacteria. Here, we found for the first time that Gram-positive bacteria naturally produce MVs into the extracellular milieu. Further characterizations showed that the density and size of *Staphylococcus aureus*-derived MVs are both similar to those of Gram-negative bacteria. With a proteomics approach, we identified with high confidence a total of 90 protein components of *S. aureus*-derived MVs. In the group of identified proteins, the highly enriched extracellular proteins suggested that a specific sorting mechanism for vesicular proteins exists. We also identified proteins that facilitate the transfer of proteins to other bacteria, as well to eliminate competing organisms, antibiotic resistance, pathological functions in systemic infections, and MV biogenesis. Taken together, these observations suggest that the secretion of MVs is an evolutionally conserved, universal process that occurs from simple organisms to complex multicellular organisms. This information will help us not only to elucidate the biogenesis and functions of MVs, but also to develop therapeutic tools for vaccines, diagnosis, and antibiotics effective against pathogenic strains of Gram-positive bacteria.

3.1381 TRAPPC2L is a Novel, Highly Conserved TRAPP-Interacting Protein
Scrivens, P.J., Shahrzad, N., Moores, A., Morin, A., Brunet, S. and Sacher, M.
Traffic, ***10(6)***, *724-736 (2009)*

Mutations in the trafficking protein particle complex C2 protein (TRAPPC2), a mammalian ortholog of yeast Trs20p and a component of the trafficking protein particle (TRAPP) vesicle tethering complex, have been linked to the skeletal disorder spondyloepiphyseal dysplasia tarda (SEDT). Intriguingly, the X-linked TRAPPC2 is just one of a complement of Trs20-related genes in humans. Here we characterize TRAPPC2L, a novel, highly conserved TRAPP-interacting protein related to TRAPPC2 and the uncharacterized yeast open reading frame *YEL048c*. TRAPPC2L and TRAPPC2 genes are found in pairs across species and show broad and overlapping expression, suggesting they are functionally distinct, a notion supported by yeast complementation studies and biochemical characterization. RNA interference-mediated knockdown of either TRAPPC2L or TRAPPC2 in HeLa cells leads to fragmentation of the Golgi, implicating both proteins in Golgi dynamics. Gradient fractionation of cellular membranes indicates that TRAPPC2L is found with a portion of cellular TRAPP on very low-density membranes whereas the remainder of TRAPP, but not TRAPPC2L, is found associated with Golgi markers. *YEL048c* displays genetic interactions with TRAPP II-encoding genes and the gene product co-fractionates with and interacts with yeast TRAPP II. Taken together these results indicate that TRAPPC2L and its yeast ortholog *YEL048c* are novel TRAPP-interacting proteins that may modulate the function of the TRAPP II complex.

3.1382 Proteomic characterization of lipid raft proteins in amyotrophic lateral sclerosis mouse spinal cord
Zhai, J., Ström, A.L., Kilty, R., Venkatakrishnan, P., White, J., Everson, W.V., Smart, E.J. and Zhu, H.
FEBS J., ***276(12)***, *3308-3323 (2009)*

Familial amyotrophic lateral sclerosis (ALS) has been linked to mutations in the copper/zinc superoxide dismutase (SOD1) gene. The mutant SOD1 protein exhibits a toxic gain-of-function that adversely affects the function of neurons. However, the mechanism by which mutant SOD1 initiates ALS is unclear. Lipid rafts are specialized microdomains of the plasma membrane that act as platforms for the organization and interaction of proteins involved in multiple functions, including vesicular trafficking, neurotransmitter signaling, and cytoskeletal rearrangements. In this article, we report a proteomic analysis using a widely used ALS mouse model to identify differences in spinal cord lipid raft proteomes between mice overexpressing wild-type (WT) and G93A mutant SOD1. In total, 413 and 421 proteins were identified in the lipid rafts isolated from WT and G93A mice, respectively. Further quantitative analysis revealed a consortium of proteins with altered levels between the WT and G93A samples. Functional classification of the 67 altered proteins revealed that the three most affected subsets of proteins were involved in: vesicular transport, and neurotransmitter synthesis and release; cytoskeletal organization and linkage to the plasma membrane; and metabolism. Other protein changes were correlated with alterations in: microglia activation and inflammation; astrocyte and oligodendrocyte function; cell signaling; cellular stress response and apoptosis; and neuronal ion channels and neurotransmitter receptor functions. Changes of selected proteins were independently validated by immunoblotting and immunohistochemistry. The significance of the lipid raft protein changes in motor neuron function and degeneration in ALS is discussed, particularly for proteins involved in vesicular trafficking and neurotransmitter signaling, and the dynamics and regulation of the plasma membrane-anchored cytoskeleton.

3.1383 Vezatin, an integral membrane protein of adherens junctions, is required for the sound resilience of cochlear hair cells

Bahloul, A., Simmler, M-C., Michel, V., Leibovici, M., Perfeettini, I., Roux, I., Weil, D., Nouaille, S., Zuo, J., Zadro, C., Licastro, D., GAsparini, P., Avan, P., Hardelin, J-P- and petit, C.
EMBO Mol. Med., ***1(2)****, 125-138 (2009)*

Loud sound exposure is a significant cause of hearing loss worldwide. We asked whether a lack of vezatin, an ubiquitous adherens junction protein, could result in noise-induced hearing loss. Conditional mutant mice bearing non-functional vezatin alleles only in the sensory cells of the inner ear (hair cells) indeed exhibited irreversible hearing loss after only one minute exposure to a 105 dB broadband sound. In addition, mutant mice spontaneously underwent late onset progressive hearing loss and vestibular dysfunction related to substantial hair cell death. We establish that vezatin is an integral membrane protein with two adjacent transmembrane domains, and cytoplasmic N- and C-terminal regions. Late recruitment of vezatin at junctions between MDCKII cells indicates that the protein does not play a role in the formation of junctions, but rather participates in their stability. Moreover, we show that vezatin directly interacts with radixin in its actin-binding conformation. Accordingly, we provide evidence that vezatin associates with actin filaments at cell–cell junctions. Our results emphasize the overlooked role of the junctions between hair cells and their supporting cells in the auditory epithelium resilience to sound trauma.

3.1384 Calcium homeostasis in plant cell nuclei

Mazars, C., Bourque, S., Mithöfer, A., Pugin, A. and Ranjeva, R.
New Phytologist, ***181(2)****, 261-274 (2009)*

In plant cells, calcium-based signaling pathways are involved in a large array of biological processes, including cell division, polarity, growth, development and adaptation to changing biotic and abiotic environmental conditions. Free calcium changes are known to proceed in a nonstereotypical manner and produce a specific signature, which mirrors the nature, strength and frequency of a stimulus. The temporal aspects of calcium signatures are well documented, but their vectorial aspects also have a profound influence on biological output. Here, we will focus on the regulation of calcium homeostasis in the nucleus. We will discuss data and present hypotheses suggesting that, while interacting with other organelles, the nucleus has the potential to generate and regulate calcium signals on its own.

3.1385 GABA receptor proteins within lipid rafts in the AY-9944 model of atypical absence seizures

Huo, J., Cortez, M.A. and Snead III, O.C.
Epilepsia, ***50(4)****, 776-788 (2009)*

Purpose: The inhibition of cholesterol synthesis with AY-9944 (AY) results in chronic recurrent atypical absence seizures in rodents. We hypothesized that cholesterol inhibition during the course of creating the AY model of atypical absence seizures results in an alteration of the entry of ϒ-aminobutyric acid $(GABA)_A$ and $GABA_B$ receptors into lipid rafts that contributes to epileptogenesis in this model.
Methods: The cholesterol synthesis inhibitor AY (7.5 mg/kg) was administered on postnatal day (P) 2, P8, P14, and P20 in Long-Evans hooded rats. The incorporation of $GABA_A$ and $GABA_B$ receptor proteins into lipid rafts of the brain was then determined.
Results: AY produced a shift of both $GABA_A$ and $GABA_B$ receptors in the examined detergent-resistant membranes (DRMs) and the soluble fractions. The percentage of the $GABA_A$ and $GABA_B$ receptors that shifted out of the DRMs varied between 17% and 50%, but the proportion of receptors in DRMs were decreased to levels around that of P5 animals or even lower. The shift observed in the AY-treated versus control animals was statistically significant ($p < 0.01$) for both $GABA_A$ and $GABA_B$ receptors.
Conclusion: Cholesterol synthesis inhibition during rat brain development that is induced by AY leads to chronic atypical absence seizures and is associated with an alteration of $GABA_A$ and $GABA_B$ receptor proteins within lipid rafts. These data suggest a novel avenue of investigation into the epileptogenesis of experimental chronic atypical absence seizures.

3.1386 Sorting defects of the tryptophan permease Tat2 in an *erg2* yeast mutant

Daicho, K., Makino, N., Hiraki, T., Ueno, M., Uritani, M., Abe, F. and Ushimaru, T.
FEMS Microbiol. Lett., ***298(2)****, 218-227 (2009)*

Cholesterol (ergosterol in yeast) in conjunction with sphingolipids forms tight-packing microdomains, 'lipid rafts,' which are thought to be critical for intracellular protein sorting in eukaryotic cells. When the activity of Erg9 involved in the first step of ergosterol biogenesis, but not that of Erg6 involved in a late step, is compromised, vacuolar degradation of the tryptophan permease Tat2 is promoted. It is unknown

whether this difference simply reflects the difference between the inhibition of early and late steps. Here, it is shown that the deletion in *ERG2*, which encodes sterol C8–C7 isomerase (the next enzymatic step after Erg6), promotes the vacuolar degradation of Tat2. It suggests that the accumulation of specific sterol intermediates may alter lipid raft structures, promoting Tat2 degradation. The *erg2*Δ-mediated Tat2 degradation required Tat2 ubiquitination. Lipid raft association of Tat2 is compromised in *erg2*Δ cells. The *erg2*Δ mutation showed a synthetic growth defect with the *trp1* mutation, indicating that Tat2 sorting is preferentially compromised in these mutants. Consistent with this notion, the raft-associated protein Pma1 was associated with detergent-resistant membranes and sorted to the plasma membrane. This study suggests the potential for the pharmacological control of cellular nutrient uptake in humans by regulating enzymes involved in cholesterol biogenesis.

3.1387 MARCKS regulates lamellipodia formation induced by IGF-I via association with PIP2 and β-actin at membrane microdomains

Yamagushi, H., Shiraishi, M., Fukami, K., Tanabe, A., Ikeda-Matsuo, Y., Naito, Y. and Sasaki, Y.
J. Cell. Physiol., ***220(3)***, *748-755 (2009)*

Myristoylated alanine-rich C kinase substrate (MARCKS) is considered to participate in formation of F-actin-based lamellipodia, which represents the first stage of neurite formation. However, the mechanism of how MARCKS is involved in lamellipodia formation is not precisely unknown. Using SH-SY5Y cells, we demonstrated here that MARCKS was translocated from cytosol to detergent-resistant membrane microdomains, known as lipid rafts, within 30 min after insulin-like growth factor-I (IGF-I) stimulation, which was accompanied by MARCKS dephosphorylation, β-actin accumulation in lipid rafts, and lamellipodia formation. The protein kinase C inhibitor, Ro-31-8220, and Rho-kinase inhibitors, HA1077 and Y27632, themselves decreased basal phosphorylation levels of MARCKS and coincidently elicited translocation of MARCKS to lipid rafts. On the other hand, the phosphoinositide 3-kinase inhibitor, LY294002, abolished IGF-I-induced dephosphorylation, translocation of MARCKS to lipid rafts, and lamellipodia formation. Treatment of cells with neomycin, a PIP2-masking reagent, attenuated the translocation of MARCKS to lipid rafts and the lamellipodia formation induced by IGF-I, although dephosphorylation of MARCKS was not affected. Immunocytochemical and immunoprecipitation analysis indicated that IGF-I stimulation induced the translocation of MARCKS to lipid rafts in the edge of lamellipodia and formation of the complex with PIP2. Moreover, we demonstrated that knockdown of endogenous MARCKS resulted in significant attenuation of IGF-I-induced β-actin accumulation in the lipid rafts and lamellipodia formation. These results suggest a novel role for MARCKS in lamellipodia formation induced by IGF-I via the translocation of MARCKS, association with PIP2, and accumulation of β-actin in the membrane microdomains.

3.1388 Compartmentalization of epidermal growth factor receptor in liver plasma membrane

Wang, Y., Posner, B.I. and Balbis, A.
J. Cell.Biochem., ***107(1)***, *96-103 (2009)*

We have investigated epidermal growth factor (EGF)-induced compartmentalization and activation of the EGF receptor (EGFR) in rat liver plasma membrane (PM) raft subfractions prepared by three different biochemical methods previously developed to characterize the composition of membrane rafts. Only detergent-resistant membranes (DRMs) possessed the basic characteristics attributed to membrane rafts. Following the administration of a low dose of EGF (1 μg/100 g BW) the content of EGFR in PM–DRMs did not change significantly; whereas after a higher dose of EGF (5 μg/100 g BW) we observed a rapid and marked disappearance of EGFR (around 80%) from both PM and DRM fractions. Interestingly, following the administration of either a low or high dose of EGF, the pool of EGFR in the PM–DRM fraction became highly Tyr-phosphorylated. In accordance with the higher level of EGFR Tyr-Phosphorylation, EGF induced an augmented recruitment of Grb2 and Shc proteins to PM–DRMs compared with whole PM. Furthermore neither high nor low doses of EGF affected the caveolin content in DRMs and PM. These observations suggest that EGFR located in DRMs are competent for signaling, and non-caveolae PM rafts are involved in the compartmentalization and internalization of the EGFR.

3.1389 Clustering transfers the translocated *Escherichia coli* receptor into lipid rafts to stimulate reversible activation of c-Fyn

Hayward, R.D., Hume, P.J., Humphreys, D., Phillips, N., Smith, K. and Koronakis, V.
Cell. Microbiol., ***11(3)***, *433-441 (2009)*

Enteropathogenic *Escherichia coli* (EPEC) mimic a ligand–receptor interaction to induce 'pedestal-like' pseudopodia on mammalian cells, providing a tractable system to study tyrosine kinase signalling to the actin cytoskeleton. EPEC delivers its own receptor (Tir), which is engaged by a bacterial surface ligand (intimin). When Tir delivery and activity are uncoupled, intimin-induced Tir clustering stimulates Tir^{Y474} phosphorylation by the Src-family kinase (SFK) c-Fyn, triggering actin polymerization and pedestal formation. How c-Fyn specifically targets Tir and is regulated remains unknown. We show that clustering transfers Tir into cholesterol-rich detergent-resistant microdomains (DRMs), a signal prompting transient c-Fyn accumulation at bacterial adhesion sites. Co-clustering of Tir^{Y474} and c-Fyn in DRMs rapidly stimulates robust kinase activation both by induced c-Fyn^{Y531} dephosphorylation to unlock the inactive state and by reciprocal c-Fyn^{Y417} autophosphorylation to promote activity. After signal induction, c-Fyn dissipates and the resting state restored by Csk-dependent phosphorylation of c-Fyn^{Y531}. These data illustrate a sophisticated mechanism evolved by a pathogen effector to reversibly regulate SFKs, and resolve early interactions at a model receptor initiating tyrosine kinase signalling.

3.1390 Vps33a Mediates RANKL Storage in Secretory Lysosomes in Osteoblastic Cells
Kariya, Y., Homma, M., Aoki, S., Chiba, A. and Suzuki, H.
J. Bone Minerald Res., ***24(10)***, *1741-1752 (2009)*

Previous studies have indicated that the amount of RANKL expressed on the cell surface of osteoblasts or bone marrow stromal cells (BMSCs) is considered an important factor determining the extent of osteoclast activation. However, subcellular trafficking of RANKL and its regulatory mechanisms in osteoblastic cells is still unclear. In this study, we showed that RANKL is predominantly localized in lysosomal organelles, but little is found on the cell surface of osteoblastic cells. We also showed that RANKL is relocated to the plasma membrane in response to stimulation with RANK-Fc–coated beads, indicating that the lysosomal organelles where RANKL is localized function as secretory lysosomes. In addition, using a protein pull-down method, we identified vacuolar protein sorting (Vps)33a as interacting with the cytoplasmic tail of RANKL. Furthermore, knockdown of Vps33a expression reduced the lysosomal storage of RANKL and caused the accumulation of newly synthesized RANKL in the Golgi apparatus, indicating that Vps33a is involved in transporting RANKL from the Golgi apparatus to secretory lysosomes. We also showed that suppression of Vps33a affects the cell surface expression level of RANKL and disrupts the regulated behavior of RANKL. These results suggest that RANKL storage in secretory lysosomes is important to control osteoclast activation and to maintain bone homeostasis.

3.1391 Overexpression of Sna3 stabilizes tryptophan permease Tat2, potentially competing for the WW domain of Rsp5 ubiquitin ligase with its binding protein Bul1
Hiraki, T. and Ebe, F.
FEBS Lett., ***584***, *55-60 (2010)*

Tryptophan permease Tat2 in *Saccharomyces cerevisiae* undergoes Rsp5-dependent degradation upon exposure to high hydrostatic pressure and it limits the growth of tryptophan auxotrophs. Overexpression of *SNA3* encoding an endosomal/vacuolar protein possessing the PPAY motif allowed growth at 25 MPa, which was potentiated by marked stabilization of Tat2. This appeared to depend on the PPAY motif, which interacted with the WW domain of Rsp5. Subcellular localization of Rsp5 was unchanged by overexpression of either *SNA3* or *SNA3-AAAY*. While the loss of Bul1, a binding protein of Rsp5, or the *rsp5-ww3* mutation allowed high-pressure growth, overexpression of *BUL1* abolished the Sna3-mediated growth at 25 MPa. These results suggest that Sna3 and Bul1 compete for the WW domain of Rsp5 upon Tat2 ubiquitination.

3.1392 The B[a]P-increased intercellular communication via translocation of connexin-43 into gap junctions reduces apoptosis
Tekpli, X., Rivedal, E., gorria, M., Landevik, N.E., Rissel, M., Dimache-Boitrel, M.T., Baffet, G., Holme, J.A. and Lagadic-Gossmann, D.
Toxicol. and Appl. Pharmacol., ***242***, *231-240 (2010)*

Gap junctions are channels in plasma membrane composed of proteins called connexins. These channels are organized in special domains between cells, and provide for direct gap junctional intercellular communication (GJIC), allowing diffusion of signalling molecules < 1 kD. GJIC regulates cell homeostasis and notably the balance between proliferation, cell cycle arrest, cell survival and apoptosis. Here, we have investigated benzo[a]pyrene (B[a]P) effects on GJIC and on the subcellular localization of the major protein of gap junction: connexin-43 (Cx43). Our results showed that B[a]P increased GJIC

between mouse hepatoma Hepa1c1c7 cells via translocation of Cx43 from Golgi apparatus and lipid rafts into gap junction plaques. Interestingly, inhibition of GJIC by chlordane or small interference RNA directed against Cx43 enhanced B[a]P-induced apoptosis in Hepa1c1c7 cells. The increased apoptosis caused by inhibition of GJIC appeared to be mediated by ERK/MAPK pathway. It is suggested that B[a]P could induce transfer of cell survival signal or dilute cell death signal via regulation of ERK/MAPK through GJIC.

3.1393 A di-arginine motif contributes to the ER localization of the type I transmembrane ER oxidoreductase TMX4

Roth, D., Lynes, E., Riemer, J., Hansen, H.G., Althaus, N., Simmen, T. And Ellgaard, L.
Biochem. J., ***425****, 195-205 (2010)*

The thiol-disulfide oxidoreductases of the PDI (protein disulfide isomerase) family assist in disulfide-bond formation in the ER (endoplasmic reticulum). In the present study, we have shown that the previously uncharacterized PDI family member TMX4 (thioredoxin-like transmembrane 4) is an N-glycosylated type I membrane protein that localizes to the ER. We also demonstrate that TMX4 contains a single ER-luminal thioredoxin-like domain, which, in contrast with similar domains in other PDIs, is mainly oxidized in living cells. The TMX4 transcript displays a wide tissue distribution, and is strongly expressed in melanoma cells. Unlike many type I membrane proteins, TMX4 lacks a typical C-terminal di-lysine retrieval signal. Instead, the cytoplasmic tail has a conserved di-arginine motif of the RXR type. We show that mutation of the RQR sequence in TMX4 to KQK interferes with ER localization of the protein. Moreover, whereas the cytoplasmic region of TMX4 confers ER localization to a reporter protein, the KQK mutant of the same protein redistributes to the cell surface. Overall, features not commonly found in other PDIs characterize TMX4 and suggest unique functional properties of the protein.

3.1394 Doppel and PrPC co-immunoprecipitate in detergent-resistant membrane domains of epithelial FRT cells

Caputo, A., Sarnataro, D., Campana, V.., Costanzo, M., Negro, A., Sorgato, M.C. and Zurzolo, C.
Biochem. J., ***425****, 341-351 (2010)*

Dpl (doppel) is a paralogue of the PrP^{C} (cellular prion protein), whose misfolded conformer (the scrapie prion protein, PrP^{Sc}) is responsible for the onset of TSEs (transmissible spongiform encephalopathies) or prion diseases. It has been shown that the ectopic expression of Dpl in the brains of some lines of PrP-knockout mice provokes cerebellar ataxia, which can be rescued by the reintroduction of the PrP gene, suggesting a functional interaction between the two proteins. It is, however, still unclear where, and under which conditions, this event may occur. In the present study we addressed this issue by analysing the intracellular localization and the interaction between Dpl and PrP^{C} in FRT (Fischer rat thyroid) cells stably expressing the two proteins separately or together. We show that both proteins localize prevalently on the basolateral surface of FRT cells, in both singly and doubly transfected clones. Interestingly we found that they associate with DRMs (detergent-resistant membranes) or lipid rafts, from where they can be co-immunoprecipitated in a cholesterol-dependent fashion. Although the interaction between Dpl and PrP^{C} has been suggested before, our results provide the first clear evidence that this interaction occurs in rafts and is dependent on the integrity of these membrane microdomains. Furthermore, both Dpl and PrP^{C} could be immunoprecipitated with flotillin-2, a raft protein involved in endocytosis and cell signalling events, suggesting that they share the same lipid environment.

3.1395 Lyn-mediated mitochondrial tyrosine phosphorylation is required to preserve mitochondrial integrity in early liver regeneration

Gringeri, E., Carraro, A., Tibald, E., D'Amico, F., Mancon, M., Toninello, A., Pagano, M.A., Vio, C., Cillo, U. and Brunati, A.M.
Biochem. J., ***425****, 401-412 (2010)*

Functional alterations in mitochondria such as overproduction of ROS (reactive oxygen species) and overloading of calcium, with subsequent change in the membrane potential, are traditionally regarded as pro-apoptotic conditions. Although such events occur in the early phases of LR (liver regeneration) after two-thirds PH (partial hepatectomy), hepatocytes do not undergo apoptosis but continue to proliferate until the mass of the liver is restored. The aim of the present study was to establish whether tyrosine phosphorylation, an emerging mechanism of regulation of mitochondrial function, participates in the response to liver injury following PH and is involved in contrasting mitochondrial pro-apoptotic signalling. Mitochondrial tyrosine phosphorylation, negligible in the quiescent liver, was detected in the early phases

of LR with a trend similar to the events heralding mitochondrial apoptosis and was attributed to the tyrosine kinase Lyn, a member of the Src family. Lyn was shown to accumulate in an active form in the mitochondrial intermembrane space, where it was found to be associated with a multiprotein complex. Our results highlight a role for tyrosine phosphorylation in accompanying, and ultimately counteracting, mitochondrial events otherwise leading to apoptosis, hence conveying information required to preserve the mitochondrial integrity during LR.

3.1396 Angiotensin-(1-7)-Angiotensin-Converting Enzyme 2 Attenuates Reactive Oxygen Species Formation to Angiotensin II Within the Cell Nucleus

Gwathmey, T.Y.M., Pendergrass, K.D., Reid, S.D., Rose, J.C., Diz, D.I. and Chappell, M.C.
*Hypertension, **55**, 166-171 (2010)*

The angiotensin (Ang) type 1 receptor (AT_1R) is highly expressed on renal nuclei and stimulates reactive oxygen species (ROS). It is not known whether other functional components of the Ang system regulate the nuclear Ang II-AT_1R ROS pathway. Therefore, we examined the expression of Ang receptors in nuclei isolated from the kidneys of young adult (1.5 years) and older adult (3.0 to 5.0 years) sheep. Binding studies in renal nuclei revealed the AT_2R as the predominant receptor subtype (≈80%) in young sheep, with the Ang-(1-7) (AT_7R; Mas protein) and AT_1R antagonists competing for the remaining sites. Conversely, in older sheep, the AT_1R accounted for ≈85% of nuclear sites, whereas the Ang type 2 receptor and AT_7R subtypes comprise ≈20% of remaining sites. Ang II increased nuclear ROS to a greater extent in older (97±22%; n=6) versus young animals (7±2%; P=0.01; n=4), and this was abolished by an AT_1R antagonist. The AT_7R antagonist D-Ala^7-Ang-(1-7) increased ROS formation to Ang II by ≈2-fold (174±5% versus 97±22%; P<0.05) in older adults. Immunoblots of renal nuclei revealed protein bands for the AT_7R and Ang-converting enzyme 2 (ACE2), which metabolizes Ang II to Ang-(1-7). The ACE2 inhibitor MLN4760 also exacerbated the Ang II-dependent formation of ROS (156±15%) and abolished the generation of Ang-(1-7) from Ang II. We conclude that an ACE2-Ang-(1-7)-AT_7R pathway modulates Ang II-dependent ROS formation within the nucleus, providing a unique protective mechanism against oxidative stress and cell damage.

3.1397 Cholesterol efflux stimulates metalloproteinase-mediated cleavage of occludin and release of extracellular membrane particles containing its C-terminal fragments

Casas, E., Barron, C., Francis, S.A., McCormack, J.M., McCarthy, K.M., Schneeberger, E.E. and Lynch, R.D.
*Exp. Cell Res., **316**, 353-365 (2010)*

That changes in membrane lipid composition alter the barrier function of tight junctions illustrates the importance of the interactions between tetraspan integral tight junction proteins and lipids of the plasma membrane. Application of methyl-β-cyclodextrin to both apical and basolateral surfaces of MDCK cell monolayers for 2 h, results in an ~80% decrease in cell cholesterol, a fall in transepithelial electrical resistance, and a 30% reduction in cell content of occludin, with a smaller reduction in levels of claudins-2, -3, and -7. There were negligible changes in levels of actin and the two non-tight junction membrane proteins GP-135 and caveolin-1. While in untreated control cells breakdown of occludin, and probably other tight junction proteins, is mediated by intracellular proteolysis, our current data suggest an alternative pathway whereby in a cholesterol-depleted membrane, levels of tight junction proteins are decreased via direct release into the intercellular space as components of membrane-bound particles. Occludin, along with two of its degradation products and several claudins, increases in the basolateral medium after incubation with methyl-β-cyclodextrin for 30 min. In contrast caveolin-1 is detected only in the apical medium after adding methyl-β-cyclodextrin. Release of occludin and its proteolytic fragments continues even after removal of methyl-β-cyclodextrin. Sedimentation and ultrastructural studies indicate that the extracellular tight junction proteins are associated with the membrane-bound particles that accumulate between adjacent cells. Disruption of the actin filament network by cytochalasin D did not diminish methyl-β-cyclodextrin-induced release of tight junction proteins into the medium, suggesting that the mechanism underlying their formation is not actin-dependent. The 41- and 48-kDa C-terminal occludin fragments formed during cholesterol depletion result from the action of a GM6001-sensitive metalloproteinase(s) at some point in the path leading to release of the membrane particles.

3.1398 α-tocopherol β-oxidation localized to rat liver mitochondria

Mustacich, D.J., Leonard, S.W., patel, N.K. and traber, M.G.
*Free Radical Biology & Medicine, **48**, 73-81 (2010)*

Approximately 40% of Americans take dietary supplements, including vitamin E (α-tocopherol). Unlike other fat-soluble vitamins, α-tocopherol is not accumulated to toxic levels. Rather tissue levels are tightly regulated, in part via increased hepatic metabolism and excretion that could, theoretically, alter metabolism of drugs, environmental toxins, and other nutrients. To date, in vivo subcellular location(s) of α-tocopherol metabolism have not been identified. The proposed pathway of α-tocopherol metabolism proceeds via ω-hydroxylation to 13′-OH-α-tocopherol, followed by successive rounds of β-oxidation to form α-CEHC. To test the hypothesis that α-tocopherol ω-hydroxylation occurs in microsomes while β-oxidation occurs in peroxisomes, rats received daily injections of vehicle, 10 mg α-tocopherol, or 10 mg trolox/100 g body wt for 3 days, and then microsomes, mitochondria, and peroxisomes were isolated from liver homogenates. Homogenate α-tocopherol levels increased 16-fold in α-tocopherol-injected rats, while remaining unchanged in trolox- or vehicle-injected rats. Total α-tocopherol recovered in the three subcellular fractions represented 93 ± 4% of homogenate α-tocopherol levels. In α-tocopherol-injected rats, microsome α-tocopherol levels increased 28-fold, while mitochondria and peroxisome levels increased 8- and 3-fold, respectively, indicating greater partitioning of α-tocopherol to the microsomes with increasing liver α-tocopherol. In α-tocopherol-injected rats, microsome 13′-OH-α-tocopherol levels increased 24-fold compared to controls, and were 7-fold greater than 13′-OH-α-tocopherol levels in peroxisome and mitochondrial fractions of α-tocopherol-injected rats. An unexpected finding was that α-CEHC, the end product of α-tocopherol metabolism, was found almost exclusively in mitochondria. These data are the first to indicate a mitochondrial role in α-tocopherol metabolism.

3.1399 Retrograde Neurotrophic Signaling Requires a Protein Interacting with Receptor Tyrosine Kinases via C2H2 Zinc Fingers

Fu, X., Zang, K., Zhou, Z., Reichardt, L.F. and Xu, B.
*Mol. Biol. Cell, **21**, 36-49 (2010)*

Neurotrophins at axonal terminals signal to cell bodies to regulate neuronal development via signaling endosomes containing activated Trk receptor tyrosine kinases and mitogen-activated protein kinases (MAPKs). Requirements for the formation of signaling endosomes remain, however, poorly characterized. Here we show that a novel Trk-interacting protein, NTRAP (neurotrophic factor receptor–associated protein), plays a crucial role in this signaling process. NTRAP interacts with the Trk intracellular domain through its C_2H_2 zinc fingers in a kinase-dependent manner. It is associated with vesicles, some of which contain markers for signaling endosomes. Inhibition of NTRAP function suppresses neurotrophin-induced neurite outgrowth in PC12 cells by altering TrkA endocytic traffic, inhibiting the formation of endosomes containing persistently active MAPKs. In compartmentalized sensory neuron cultures, down-regulation of NTRAP abolishes the ability of neurotrophins applied to distal axons to activate the transcription factor adenosine 3',5'-monophosphate response element-binding protein (CREB) and to promote neuronal survival. We propose that NTRAP regulates retrograde neurotrophic signaling by controlling the formation of signaling endosomes.

3.1400 Sec3-containing Exocyst Complex Is Required for Desmosome Assembly in Mammalian Epithelial Cells

Andersen, N.J. and Yeaman, C.
*Mol. Biol. Cell, **21**, 152-164 (2010)*

The Exocyst is a conserved multisubunit complex involved in the docking of post-Golgi transport vesicles to sites of membrane remodeling during cellular processes such as polarization, migration, and division. In mammalian epithelial cells, Exocyst complexes are recruited to nascent sites of cell–cell contact in response to E-cadherin–mediated adhesive interactions, and this event is an important early step in the assembly of intercellular junctions. Sec3 has been hypothesized to function as a spatial landmark for the development of polarity in budding yeast, but its role in epithelial cells has not been investigated. Here, we provide evidence in support of a function for a Sec3-containing Exocyst complex in the assembly or maintenance of desmosomes, adhesive junctions that link intermediate filament networks to sites of strong intercellular adhesion. We show that Sec3 associates with a subset of Exocyst complexes that are enriched at desmosomes. Moreover, we found that membrane recruitment of Sec3 is dependent on cadherin-mediated adhesion but occurs later than that of the known Exocyst components Sec6 and Sec8 that are recruited to adherens junctions. RNA interference-mediated suppression of Sec3 expression led to specific impairment of both the morphology and function of desmosomes, without noticeable effect on adherens junctions. These results suggest that two different exocyst complexes may function in basal–lateral membrane trafficking and will enable us to better understand how exocytosis is spatially organized during development of epithelial plasma membrane domains.

3.1401 HIV Nef is Secreted in Exosomes and Triggers Apoptosis in Bystander CD4+ T Cells
Lenassi, M., Cagney, G., Liao, M., Vaupotic, T., Bartholomeeusen, K., Cheng, Y., Krogan, N.J., Plemenitas, A. and Peterlin, B.M.
Traffic, ***11***, *110-122 (2010)*

The HIV accessory protein negative factor (Nef) is one of the earliest and most abundantly expressed viral proteins. It is also found in the serum of infected individuals (Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol 2005;17:879-887). Extracellular Nef protein has deleterious effects on CD4+ T cells (James CO, Huang MB, Khan M, Garcia-Barrio M, Powell MD, Bond VC. Extracellular Nef protein targets CD4+ T cells for apoptosis by interacting with CXCR4 surface receptors. J Virol 2004;78:3099-3109), the primary targets of HIV, and can suppress immunoglobulin class switching in bystander B cells (Qiao X, He B, Chiu A, Knowles DM, Chadburn A, Cerutti A. Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. Nat Immunol 2006;7:302-310). Nevertheless, the mode of exit of Nef from infected cells remains a conundrum. We found that Nef stimulates its own export via the release of exosomes from all cells examined. Depending on its intracellular location, these Nef exosomes form at the plasma membrane, late endosomes or both compartments in Jurkat, SupT1 and primary T cells, respectively. Nef release through exosomes is conserved also during HIV-1 infection of peripheral blood lymphocytes (PBLs). Released Nef exosomes cause activation-induced cell death of resting PBLs in vitro. Thus, HIV-infected cells export Nef in bioactive vesicles, which facilitate the depletion of CD4+ T cells that is a hallmark of acquired immunodeficiency syndrome (AIDS).

3.1402 How Do Bone Cells Secrete Proteins?
Zhao, H., Ito, Y., Chappel, J., Andrews, N., Ross, F.P. and Teitelbaum, S.L.
Adv. In Exp. Med. And Biol., ***658***, *105-109 (2010)*

Osteoclasts (OCs), which are the exclusive bone resorbing cells, degrade skeletal matrix by forming an intimate relationship with the bone surface. Thus, when OCs attach to bone, they produce an actin-rich sealing zone representing a gasket-like structure, which isolates the resorptive milieu from the general extracellular space. This “resorptive microenvironment” contains a ruffled border, the unique bone-degrading organelle of the OC, which consists of a complex, villous-like organization of the plasma membrane. This structure appears only in resorbing cells and is the product of signals derived from the bone matrix. These signals polarize as yet undefined acidified vesicles containing the OC vacuolar H+ATPase towards the bone-apposed plasma membrane, into which they insert, thereby increasing its complexity. The ruffled border is thus the most definitive marker of the resorbing osteoclast.

3.1403 The Mycobacterium bovis Bacille Calmette-Guérin Phagosome Proteome
Lee, B-Y., Jethwaney, D., Schilling, B., Clemens, D.L., Gibson, B.W. and Horwitz, M.A.
Mol. Cell. Proteomics, ***9(1)***, *32-53 (2010)*

Mycobacterium tuberculosis and *Mycobacterium bovis* bacille Calmette-Guérin (BCG) alter the maturation of their phagosomes and reside within a compartment that resists acidification and fusion with lysosomes. To define the molecular composition of this compartment, we developed a novel method for obtaining highly purified phagosomes from BCG-infected human macrophages and analyzed the phagosomes by Western immunoblotting and mass spectrometry-based proteomics. Our purification procedure revealed that BCG grown on artificial medium becomes less dense after growth in macrophages. By Western immunoblotting, LAMP-2, Niemann-Pick protein C1, and syntaxin 3 were readily detectable on the BCG phagosome but at levels that were lower than on the latex bead phagosome; flotillin-1 and the vacuolar ATPase were barely detectable on the BCG phagosome but highly enriched on the latex bead phagosome. Immunofluorescence studies confirmed the scarcity of flotillin on BCG phagosomes and demonstrated an inverse correlation between bacterial metabolic activity and flotillin on *M. tuberculosis* phagosomes. By mass spectrometry, 447 human host proteins were identified on BCG phagosomes, and a partially overlapping set of 289 human proteins on latex bead phagosomes was identified. Interestingly, the majority of the proteins identified consistently on BCG phagosome preparations were also identified on latex bead phagosomes, indicating a high degree of overlap in protein composition of these two compartments. It is likely that many differences in protein composition are quantitative rather than qualitative in nature. Despite the remarkable overlap in protein composition, we consistently identified a number of proteins on the BCG phagosomes that were not identified in any of our latex bead phagosome preparations, including proteins involved in membrane trafficking and signal transduction, such as Ras GTPase-activating-like

protein IQGAP1, and proteins of unknown function, such as FAM3C. Our phagosome purification procedure and initial proteomics analyses set the stage for a quantitative comparative analysis of mycobacterial and latex bead phagosome proteomes.

3.1404 Peroxisomes from the Heavy Mitochondrial Fraction: Isolation by Zonal Free Flow Electrophoresis and Quantitative Mass Spectrometrical Characterization
Islinger, M., Li, K.W., Loos, M., Liebler, S., Angermüller, S., Eckerskorn, C., Weber, G., Abdolzade, A. and Völkl, A.
J. Proteome Res., ***9***, *113-124 (2010)*

Peroxisomes are a heterogeneous group of organelles fulfilling reactions in a variety of metabolic pathways. To investigate if functionally different subpopulations can be found within a single tissue, peroxisomes from the heavy mitochondrial fraction (HM-Po) of the rat liver were isolated and compared to "classic" peroxisomes from the light mitochondrial fraction (LM-Po) using iTRAQ tandem mass spectrometry. Peroxisomes represent only a minor although significant proportion of the heavy mitochondrial fraction ($2700g_{max}$) precluding a straightforward isolation by standard protocols. Thus, a new fractionation scheme suitable for a subsequent mass spectrometrical analysis was developed using a combination of centrifugation techniques and zonal free flow electrophoresis. On the basis of the iTRAQ-measurement, a variation of the peroxisomal protein pattern between both fractions could be determined and further confirmed by immunoblotting and enzyme activity assays for selected proteins: whereas peroxisomes from the light mitochondrial fraction contain high amounts of β-oxidation enzymes, peroxisomes from the heavy mitochondrial fraction were dominated by enzymes fulfilling other functions. Among other findings, HM-Po was characterized by a high abundance of D-amino acid oxidase. This observation can be mirrored at the ultrastructural level, where tissue sections of liver peroxisomes show a heterogeneous staining for the enzymes activity, when visualized by the cerium technique.

3.1405 Adeno-associated Virus Gene Therapy With Cholesterol 24-Hydroxylase Reduces the Amyloid Pathology Before or After the Onset of Amyloid Plaques in Mouse Models of Alzheimer's Disease
Hudry,, E., Van Dam, D., Kulik, W., De Deyn, P.P., Stety, F.S., Ahouansou, O., Benraiss, A., Delacourte, A., Bougneres, P., Aubourg, P. and Cartier, N.
Molecular Therapy, ***18(1)***, *44-53 (2010)*

The development of Alzheimer's disease (AD) is closely connected with cholesterol metabolism. Cholesterol increases the production and deposition of amyloid-β (Aβ) peptides that result in the formation of amyloid plaques, a hallmark of the pathology. In the brain, cholesterol is synthesized *in situ* but cannot be degraded nor cross the blood–brain barrier. The major exportable form of brain cholesterol is 24S-hydroxycholesterol, an oxysterol generated by the neuronal cholesterol 24-hydroxylase encoded by the *CYP46A1* gene. We report that the injection of adeno-associated vector (AAV) encoding *CYP46A1* in the cortex and hippocampus of APP23 mice before the onset of amyloid deposits markedly reduces Aβ peptides, amyloid deposits and trimeric oligomers at 12 months of age. The Morris water maze (MWM) procedure also demonstrated improvement of spatial memory at 6 months, before the onset of amyloid deposits. AAV5-wtCYP46A1 vector injection in the cortex and hippocampus of amyloid precursor protein/presenilin 1 (APP/PS) mice after the onset of amyloid deposits also reduced markedly the number of amyloid plaques in the hippocampus, and to a less extent in the cortex, 3 months after the injection. Our data demonstrate that neuronal overexpression of *CYP46A1* before or after the onset of amyloid plaques significantly reduces Aβ pathology in mouse models of AD.

3.1406 Cathepsin B-mediated Autophagy Flux Facilitates the Anthrax Toxin Receptor 2-mediated Delivery of Anthrax Lethal Factor into the Cytoplasm
Ha, S-D., Ham, B., Mogridge, J., Saftig, P., Lin, S. and Kim, S.O.
J. Biol. Chem., ***285(3)***, *2120-2129 (2010)*

Anthrax lethal toxin (LeTx) is a virulence factor secreted by Bacillus anthracis and has direct cytotoxic effects on most cells once released into the cytoplasm. The cytoplasmic delivery of the proteolytically active component of LeTx, lethal factor (LF), is carried out by the transporter component, protective antigen, which interacts with either of two known surface receptors known as anthrax toxin receptor (ANTXR) 1 and 2. We found that the cytoplasmic delivery of LF by ANTXR2 was mediated by cathepsin

B (CTSB) and required lysosomal fusion with LeTx-containing endosomes. Also, binding of protective antigen to ANXTR1 or -2 triggered autophagy, which facilitated the cytoplasmic delivery of ANTXR2-associated LF. We found that whereas cells treated with the membrane-permeable CTSB inhibitor CA074-Me- or CTSB-deficient cells had no defect in fusion of LC3-containing autophagic vacuoles with lysosomes, autophagic flux was significantly delayed. These results suggested that the ANTXR2-mediated cytoplasmic delivery of LF was enhanced by CTSB-dependent autophagic flux.

3.1407 CD147, a γ-secretase associated protein is upregulated in Alzheimer's disease brain and its cellular trafficking is affected by presenilin-2
Nahalkova, J., Volk,mann, I., Aoki, M., Winblad, B., Bogdanovic, N., Tjernberg, L.O. and Behbahani, H.
*Neurochem. Int., **56**, 67-76 (2010)*

γ-Secretase activity has been extensively investigated due to its role in Alzheimer's disease. Here, we studied the association of CD147, a transmembrane glycoprotein belonging to the immunoglobulin family, with γ-secretase and its expression in Alzheimer's disease and control tissues. Subcellular fractionation of postmitochondrial supernatant from rat brain on step iodixanol gradient in combination with co-immunoprecipitation using an anti-nicastrin antibody showed association of limited amount of CD147 to γ-secretase. By immunoblotting of postnuclear pellets from Alzheimer's disease and control human brain tissues we showed that CD147 with molecular weight 75 kDa is upregulated in frontal cortex and thalamus of the Alzheimer's disease brains. Immunohistochemistry of brain tissues from Alzheimer's disease and control revealed specific upregulation of CD147 in neurons, axons and capillaries of Alzheimer's disease frontal cortex and thalamus. The effect of presenilin-1 and -2, which are the catalytic subunits of γ-secretase, on CD147 expression and subcellular localization was analyzed by confocal microscopy in combination with flow cytometry and showed that PS2 affected the subcellular localization of CD147 in mouse embryonic fibroblast cells. We suggest that a small fraction of CD147 present in the brain is associated with the γ-secretase, and can be involved in mechanisms dysregulated in Alzheimer's disease brain.

3.1408 The cytoplasmic tail of fibrocystin contains a ciliary targeting sequence
Follit, J.A., Li, L., Vucica, Y and Pazour, G.J:
*J. Cell Biol., **188(1)**, 21-28 (2010)*

Sensory functions of primary cilia rely on ciliary-localized membrane proteins, but little is known about how these receptors are targeted to the cilium. To further our understanding of this process, we dissected the ciliary targeting sequence (CTS) of fibrocystin, the human autosomal recessive polycystic kidney disease gene product. We show that the fibrocystin CTS is an 18-residue motif localized in the cytoplasmic tail. This motif is sufficient to target green fluorescent protein (GFP) to cilia of ciliated cells and targets GFP to lipid rafts if the cells are not ciliated. Rab8, but not several other Rabs implicated in ciliary assembly, binds to the CTS in a coimmunoprecipitation assay. Dominant-negative Rab8 interacts more strongly than wild-type or constitutively active Rab8, and coexpression of this dominant-negative mutant Rab8 blocks trafficking to the cilium. This suggests that the CTS functions by binding regulatory proteins like Rab8 to control trafficking through the endomembrane system and on to the cilium.

3.1409 WAVE1 regulates Bcl-2 localization and phosphorylation in leukemia cells
Kang, R., Tang, D., Yu, Y., Wang, Z., Hu, T., Wang, H. and Cao, L.
*Leukemia, **24**, 177-186 (2010)*

Bcl-2 proteins are over-expressed in many tumors and are critically important for cell survival. Their anti-apoptotic activities are determined by intracellular localization and post-translational modifications (such as phosphorylation). Here, we showed that WAVE1, a member of the Wiskott–Aldrich syndrome protein family, was over-expressed in blood cancer cell lines, and functioned as a negative regulator of apoptosis. Further enhanced expression of WAVE1 by gene transfection rendered leukemia cells more resistant to anti-cancer drug-induced apoptosis; whereas suppression of WAVE1 expression by RNA interference restored leukemia cells' sensitivity to anti-drug-induced apoptosis. WAVE1 was found to be associated with mitochondrial Bcl-2, and its depletion led to mitochondrial release of Bcl-2, and phosphorylation of ASK1/JNK and Bcl-2. Furthermore, depletion of WAVE1 expression increased anti-cancer drug-induced production of reactive oxygen species in leukemia cells. Taken together, these results suggest WAVE1 as a novel regulator of apoptosis, and potential drug target for therapeutic intervention of leukemia.

3.1410 UTP Controls Cell Surface Distribution and Vasomotor Activity of the Human P2Y2 Receptor through an Epidermal Growth Factor Receptor-transregulated Mechanism

Norambuena, A., Palma, F., Poblete, M.I., Domoso, M.V., Pardo, E., Gonzalez, A. and Huidobro-Toro, J.P.
J. Biol. Chem., ***285(5)***, *2940-2950 (2010)*

Extracellular nucleotides transmit signals into the cells through the P2 family of cell surface receptors. These receptors are amply expressed in human blood vessels and participate in vascular tone control; however, their signaling mechanisms remain unknown. Here we show that in smooth muscle cells of isolated human chorionic arteries, the activation of the $P2Y_2$ receptor ($P2Y_2R$) induces not only its partition into membrane rafts but also its rapid internalization. Cholesterol depletion with methyl-β-cyclodextrin reduced the association of the agonist-activated receptor into membrane rafts but did not affect either the UTP-mediated vasoconstrictions or the vasomotor responses elicited by both serotonin and KCl. *Ex vivo* perfusion of human chorionic artery segments with 1–10 μM UTP, a selective $P2Y_2R$ agonist, displaced the $P2Y_2R$ localization into membrane rafts within 1 min, a process preceded by the activation of both RhoA and Rac1 GTPases. AG1478, a selective and potent inhibitor of the epidermal growth factor receptor tyrosine kinase activity, not only blocked the UTP-induced vasomotor activity but also abrogated both RhoA and Rac1 activation, the $P2Y_2R$ association with membrane rafts, and its internalization. Altogether, these results show for the first time that the plasma membrane distribution of the $P2Y_2R$ is transregulated by the epidermal growth factor receptor, revealing an unsuspected functional interplay that controls both the membrane distribution and the vasomotor activity of the $P2Y_2R$ in intact human blood vessels.

3.1411 Differentiation of human adipose-derived stem cells induced by recombinantly expressed fibroblast growth factor 10 in vitro and in vivo

Zhang, X., Wu, M., Zhang, W., Shen, J. and Liu, H.
In vitro Cell. Dev. Biol. – Animal, ***46***, *60-71 (2010)*

The adipogenesis effect of fibroblast growth factor 10 (FGF10) has been demonstrated in many studies. The aim of this study is to render a novel method which can continuously induce hypodermal adipose-derived stem cell (ADSC) differentiation and maturation in vivo and in vitro using FGF10. We constructed a recombinant pcDNA3.0-FGF10-MSC which can continuously express FGF10 by transfected FGF10 into a human mesenchymal stem cell (MSC) clone, and we cultured ADSCs from human subcutaneous resected adipose tissue. An in vitro and in vivo co-culture system of pcDNA3.0-FGF10-MSC and ADSCs was then established. We observed the characteristics of ADSCs, monitored the adipogenesis-related transcription factor CAAT/enhancer binding protein-β, peroxisome proliferator-activated receptor-γ, and measured the adipose tissue layer of carrier animals. The results showed that FGF10 secreted from pcDNA3.0-FGF10-MSC could induce ADSC differentiation into mature adipocytes consistently. The study demonstrated that FGF10 can promote the adipogenesis effect in situ, and the autotransplantation of a carrier continuously secreting FGF10 may be utilized for increasing local subcutaneous adipose tissue in cosmetology.

3.1412 Mitochondrial Localization of Vitamin D Receptor in Human Platelets and Differentiated Megakaryocytes

Silvagno, F., De Vivo, E., Attanasio, A., gallo, V., Mazzucco, G. and Pescamona, G.
PloSOne, ***5(1)***, *e8670 (2010)*

Background
Like other steroid hormones, vitamin D elicits both transcriptional events and rapid non genomic effects. Vitamin D receptor (VDR) localization and mechanisms of VDR-triggered non genomic responses are still controversial. Although anticoagulant effects of vitamin D have been reported and VDR signalling has been characterized in monocytes and vascular cells, nothing is known about VDR expression and functions in human platelets, anucleated fragments of megakaryocytes which are known targets of other steroids.
Methodology/Principal Findings
In this study we characterized the expression and cellular localization of VDR in human platelets and in a megakaryocyte lineage. Human platelets and their TPA-differentiated precursors expressed a classical 50 kDa VDR protein, which increased with megakaryocytes maturation. By biochemical fractionation studies we demonstrated the presence of the receptor in the soluble and mitochondrial compartment of human platelets, and the observation was confirmed by immunoelectron microscopy analysis. Similar localization was found in mature megakaryocytes, where besides its classical nuclear localization the receptor was evident as soluble and mitochondria resident protein.
Conclusions

The results reported here suggest that megakaryocytopoiesis and platelet activation, which are calcium-dependent events, might be modulated by a mitochondrial non genomic activity of VDR. These data open challenging future studies on VDR physiological role in platelets and more generally in mitochondria.

3.1413 Synaptic and Endosomal Localization of Active γ-Secretase in Rat Brain

Frykman, S., Hur, J-Y., Frånberg, J., Aoki, M., Winblad, B., Nahalkova, J., Behbahani, H. and Tjernberg, L.O.

*PloSOne, **5(1)**, e8948 (2010)*

Background

A key player in the development of Alzheimer's disease (AD) is the γ-secretase complex consisting of at least four components: presenilin, nicastrin, Aph-1 and Pen-2. γ-Secretase is crucial for the generation of the neurotoxic amyloid β-peptide (Aβ) but also takes part in the processing of many other substrates. In cell lines, active γ-secretase has been found to localize primarily to the Golgi apparatus, endosomes and plasma membranes. However, no thorough studies have been performed to show the subcellular localization of the active γ-secretase in the affected organ of AD, namely the brain.

Principal Findings

We show by subcellular fractionation of rat brain that high γ-secretase activity, as assessed by production of Aβ40, is present in an endosome- and plasma membrane-enriched fraction of an iodixanol gradient. We also prepared crude synaptic vesicles as well as synaptic membranes and both fractions showed high Aβ40 production and contained high amounts of the γ-secretase components. Further purification of the synaptic vesicles verified the presence of the γ-secretase components in these compartments. The localization of an active γ-secretase in synapses and endosomes was confirmed in rat brain sections and neuronal cultures by using a biotinylated γ-secretase inhibitor together with confocal microscopy.

Significance

The information about the subcellular localization of γ-secretase in brain is important for the understanding of the molecular mechanisms of AD. Furthermore, the identified fractions can be used as sources for highly active γ-secretase.

3.1414 Proteomics Analysis of A33 Immunoaffinity-purified Exosomes Released from the Human Colon Tumor Cell Line LIM1215 Reveals a Tissue-specific Protein Signature

Mathivanan, S., Lim, J.W.E., Tauro, B.J., Ji, H., Moritz, R.L. and Simpson, R.

*Mol. Cell. Protemics, **9(2)**, 197-208 (2010)*

Exosomes are 40–100-nm-diameter nanovesicles of endocytic origin that are released from diverse cell types. To better understand the biological role of exosomes and to avoid confounding data arising from proteinaceous contaminants, it is important to work with highly purified material. Here, we describe an immunoaffinity capture method using the colon epithelial cell-specific A33 antibody to purify colorectal cancer cell (LIM1215)-derived exosomes. LC-MS/MS revealed 394 unique exosomal proteins of which 112 proteins (28%) contained signal peptides and a significant enrichment of proteins containing coiled coil, RAS, and MIRO domains. A comparative protein profiling analysis of LIM1215-, murine mast cell-, and human urine-derived exosomes revealed a subset of proteins common to all exosomes such as endosomal sorting complex required for transport (ESCRT) proteins, tetraspanins, signaling, trafficking, and cytoskeletal proteins. A conspicuous finding of this comparative analysis was the presence of host cell-specific (LIM1215 exosome) proteins such as A33, cadherin-17, carcinoembryonic antigen, epithelial cell surface antigen (EpCAM), proliferating cell nuclear antigen, epidermal growth factor receptor, mucin 13, misshapen-like kinase 1, keratin 18, mitogen-activated protein kinase 4, claudins (1, 3, and 7), centrosomal protein 55 kDa, and ephrin-B1 and -B2. Furthermore, we report the presence of the enzyme phospholipid scramblase implicated in transbilayer lipid distribution membrane remodeling. The LIM1215-specific exosomal proteins identified in this study may provide insights into colon cancer biology and potential diagnostic biomarkers.

3.1415 Application of Proteomic Marker Ensembles to Subcellular Organelle Identification

Andreyev, A.Y., Shen, Z., Guan, Z., Ryan, A., Fahy, E., Subramaniam, S., Raetz, C.R.H., Briggs, S. and Dennis, E.A.

*Mol. Cell. Proteomics, **9(2)**, 388-402 (2010)*

Compartmentalization of biological processes and the associated cellular components is crucial for cell function. Typically, the location of a component is revealed through a co-localization and/or co-purification with an organelle marker. Therefore, the identification of reliable markers is critical for a thorough

understanding of cellular function and dysfunction. We fractionated macrophage-like RAW264.7 cells, both in the resting and endotoxin-activated states, into six fractions representing the major organelles/compartments: nuclei, mitochondria, cytoplasm, endoplasmic reticulum, and plasma membrane as well as an additional dense microsomal fraction. The identity of the first five of these fractions was confirmed via the distribution of conventional enzymatic markers. Through a quantitative liquid chromatography/mass spectrometry-based proteomics analysis of the fractions, we identified 50-member ensembles of marker proteins ("marker ensembles") specific for each of the corresponding organelles/compartments. Our analysis attributed 206 of the 250 marker proteins (~82%) to organelles that are consistent with the location annotations in the public domain (obtained using DAVID 2008, EntrezGene, Swiss-Prot, and references therein). Moreover, we were able to correct locations for a subset of the remaining proteins, thus proving the superior power of analysis using multiple organelles as compared with an analysis using one specific organelle. The marker ensembles were used to calculate the organelle composition of the six above mentioned subcellular fractions. Knowledge of the precise composition of these fractions can be used to calculate the levels of metabolites in the pure organelles. As a proof of principle, we applied these calculations to known mitochondria-specific lipids (cardiolipins and ubiquinones) and demonstrated their exclusive mitochondrial location. We speculate that the organelle-specific protein ensembles may be used to systematically redefine originally morphologically defined organelles as biochemical entities.

3.1416 Chromogranin B Gene Ablation Reduces the Catecholamine Cargo and Decelerates Exocytosis in Chromaffin Secretory Vesicles

Diaz-Vera, J., Morales, Y.G., Hernandez-Fernaud, J.R., Camacho, M., Montesinos, M.S., Calegari, F., Huttner, W.B., Borges, R. and Machado, J.D.
J. Neurosci., ***30(3)****, 950-957 (2010)*

Chromogranins/secretogranins (Cgs) are the major soluble proteins of large dense-core secretory vesicles (LDCVs). We have recently reported that the absence of chromogranin A (CgA) caused important changes in the accumulation and in the exocytosis of catecholamines (CAs) using a CgA-knock-out (CgA-KO) mouse. Here, we have analyzed a CgB-KO mouse strain that can be maintained in homozygosis. These mice have 36% less adrenomedullary epinephrine when compared to $Chgb^{+/+}$ [wild type (WT)], whereas the norepinephrine content was similar. The total evoked release of CA was 33% lower than WT mice. This decrease was not due to a lower frequency of exocytotic events but to less secretion per quantum (~ 30%) measured by amperometry; amperometric spikes exhibited a slower ascending but a normal decaying phase. Cell incubation with L-DOPA increased the vesicle CA content of WT but not of the CgB-KO cells. Intracellular electrochemistry, using patch amperometry, showed that L-DOPA overload produced a significantly larger increase in cytosolic CAs in cells from the KO animals than chromaffin cells from the WT. These data indicate that the mechanisms for vesicular accumulation of CAs in the CgB-KO cells were saturated, while there was ample capacity for further accumulation in WT cells. Protein analysis of LDCVs showed the overexpression of CgA as well as other proteins apparently unrelated to the secretory process. We conclude that CgB, like CgA, is a highly efficient system directly involved in monoamine accumulation and in the kinetics of exocytosis from LDCVs.

3.1417 The Ubiquitin–Proteasome System Regulates the Stability of Neuronal Nicotinic Acetylcholine Receptors

Rezvani, K., Teng, Y. and De Biasi, M.
J. Mol. Neurosci., ***40****, 177-184 (2010)*

Ubiquitination is a key event for protein degradation by the proteasome system, membrane protein internalization, and protein trafficking among cellular compartments. Few data are available on the role of the ubiquitin–proteasome system (UPS) in the trafficking of neuronal nicotinic acetylcholine receptors (nAChRs). Experiments conducted in neuron-like differentiated rat pheochromocytoma cells (PC12 cells) show that the α3, β2, and β4 nAChR subunits are ubiquitinated and that their ubiquitination is necessary for degradation. A 24-h treatment with the proteasome inhibitor PS-341 increased the total levels of α3 and the two β subunits in both whole cell lysates and fractions enriched for the ER/Golgi compartment. nAChR subunit upregulation was also detected in plasma membrane-enriched fractions. Inhibition of the lysosomal degradation machinery by E-64 had a significantly smaller effect on nAChR turnover. The present data, together with previous results showing that the α7 nAChR subunit is a target of the UPS, point to a prominent role of the proteasome in nAChR trafficking.

3.1418 Lovastatin-induced cholesterol depletion affects both apical sorting and endocytosis of aquaporin-2 in renal cells

Procino, G., Barbieri, C., carmosino, M., Rizzo, F., Valenti, G. and Svelto, M.
*Am. J. Physiol. Renal Physiol., **298**, F266-F278 (2010)*

Vasopressin causes the redistribution of the water channel aquaporin-2 (AQP2) from cytoplasmic storage vesicles to the apical plasma membrane of collecting duct principal cells, leading to urine concentration. The molecular mechanisms regulating the selective apical sorting of AQP2 are only partially uncovered. In this work, we investigate whether AQP2 sorting/trafficking is regulated by its association with membrane rafts. In both MCD4 cells and rat kidney, AQP2 preferentially associated with Lubrol WX-insoluble membranes regardless of its presence in the storage compartment or at the apical membrane. Block-and-release experiments indicate that *1*) AQP2 associates with detergent-resistant membranes early in the biosynthetic pathway; *2*) strong cholesterol depletion delays the exit of AQP2 from the *trans*-Golgi network. Interestingly, mild cholesterol depletion promoted a dramatic accumulation of AQP2 at the apical plasma membrane in MCD4 cells in the absence of forskolin stimulation. An internalization assay showed that AQP2 endocytosis was clearly reduced under this experimental condition. Taken together, these data suggest that association with membrane rafts may regulate both AQP2 apical sorting and endocytosis.

3.1419 Endocytic Rab proteins are required for hepatitis C virus replication complex formation

Manna, D., Aligo, J., Xu, C., Park, W.S., Koc, H., Heo, W.D. and Konan, K.V.
*Virology, **398**, 21-37 (2010)*

During infection, hepatitis C virus (HCV) NS4B protein remodels host membranes to form HCV replication complexes (RC) which appear as foci under fluorescence microscopy (FM). To understand the role of Rab proteins in forming NS4B foci, cells expressing the HCV replicon were examined biochemically and via FM. First, we show that an isolated NS4B-bound subcellular fraction is competent for HCV RNA synthesis. Further, this fraction is differentially enriched in Rab1, 2, 5, 6 and 7. However, when examined via FM, NS4B foci appear to be selectively associated with Rab5 and Rab7 proteins. Additionally, dominant negative (DN) Rab6 expression impairs Rab5 recruitment into NS4B foci. Further, silencing of Rab5 or Rab7 resulted in a significant decrease in HCV genome replication. Finally, expression of DN Rab5 or Rab7 led to a reticular NS4B subcellular distribution, suggesting that endocytic proteins Rab5 and Rab7, but not Rab11, may facilitate NS4B foci formation.

3.1420 Cysteinyl leukotrienes acting via granule membrane-expressed receptors elicit secretion from within cell-free human eosinophil granules

Neves, J., Radke, A.L. and Weller, P.F.
*J. Allergy Clin. Immunol., **125**, 477-482 (2010)*

Background
Cysteinyl leukotrienes (cysLTs) are recognized to act via receptors (cysLTRs) expressed on cell surface plasma membranes. Agents that block $cysLT_1$ receptor ($cysLT_1R$) are therapeutics for allergic disorders. Eosinophils contain multiple preformed proteins stored within their intracellular granules. Cell-free eosinophil granules are present extracellularly as intact membrane-bound organelles in sites associated with eosinophil infiltration, including asthma, rhinitis, and urticaria, but have unknown functional capabilities.
Objective
We evaluated the expression of cysLTRs on eosinophil granule membranes and their functional roles in eliciting protein secretion from within eosinophil granules.
Methods
We studied secretory responses of human eosinophil granules isolated by subcellular fractionation. Granules were stimulated with cysLTs, and eosinophil cationic protein and cytokines were measured in the supernatants. Receptor expression on granule membranes and eosinophils was evaluated by flow cytometry and Western blot.
Results
We report that receptors for cysLTs, $cysLT_1R$, $cysLT_2$ receptor, and the purinergic P2Y12 receptor, are expressed on eosinophil granule membranes. Leukotriene (LT) C_4 and extracellularly generated LTD_4 and LTE_4 stimulated isolated eosinophil granules to secrete eosinophil cationic protein. MRS 2395, a P2Y12 receptor antagonist, inhibited cysLT-induced eosinophil cationic protein release. Montelukast, likely not solely as an inhibitor of $cysLT_1R$, inhibited eosinophil cationic protein release elicited by LTC_4 and LTD_4 as well as by LTE_4.

Conclusion
These studies identify previously unrecognized sites of localization, the membranes of intracellular eosinophil granule organelles, and function for cysLT-responsive receptors that mediate cysteinyl leukotriene-stimulated secretion from within eosinophil granules, including those present extracellularly.

3.1421 Local translation of dendritic *RhoA* revealed by an improved synaptoneurosome preparation
Troca-Marin, J.A., Alves-Sampio, A., Tejedor, F.J. and Montesinos, M.L.
Mol. Cell. Neurosci., ***43***, *308-314 (2010)*

Changes in dendritic spine morphology, a hallmark of synaptic plasticity, involve remodeling of the actin cytoskeleton, a process that is regulated by Rho GTPases. RhoA, a member of this GTPase family, segregates to dendrites in differentiated neurons. Given the emerging role of dendritic mRNA local translation in synaptic plasticity, we have assessed the possible localization and translation of *RhoA* mRNA at dendrites. At this end, we have developed and describe here in detail an improved method for isolating hippocampal and neocortical mouse synaptoneurosomes. This synaptoneurosomal preparation is much more enriched in synaptic proteins than those obtained in former methods, exhibits *bona fide* electron microscopy pre- and postsynaptic morphologies, contains abundant dendritic mRNAs, and is competent for activity-regulated protein synthesis. Using this preparation, we have found that *RhoA* mRNA is dendritically localized and its local translation is enhanced by BDNF stimulation. These findings suggest that some of the known functions of RhoA on spine morphology may be mediated by regulating its local translation.

3.1422 Cadherins and Pak1 Control Contact Inhibition of Proliferation by Pak1-βPIX-GIT Complex-Dependent Regulation of Cell-Matrix Signaling
Liu, F., Jia, L., Thompson-Baine, A-M. Puglise, J.M. ter Beest, M.B.A. and Zegers, M.M.P.
Mol. Cell. Biol., ***30(8)***, *1971-1983 (2010)*

It is crucial for organ homeostasis that epithelia have effective mechanisms to restrict motility and cell proliferation in order to maintain tissue architecture. On the other hand, epithelial cells need to rapidly and transiently acquire a more mesenchymal phenotype, with high levels of cell motility and proliferation, in order to repair epithelia upon injury. Cross talk between cell-cell and cell-matrix signaling is crucial for regulating these transitions. The Pak1-βPIX-GIT complex is an effector complex downstream of the small GTPase Rac1. We previously showed that translocation of this complex from cell-matrix to cell-cell adhesion sites was required for the establishment of contact inhibition of proliferation. In this study, we provide evidence that this translocation depends on cadherin function. Cadherins do not recruit the complex by direct interaction. Rather, we found that inhibition of the normal function of cadherin or Pak1 leads to defects in focal adhesion turnover and to increased signaling by phosphatidylinositol 3-kinase. We propose that cadherins are involved in regulation of contact inhibition by controlling the function of the Pak1-βPIX-GIT complex at focal contacts.

3.1423 Cell Signaling, Internalization, and Nuclear Localization of the Angiotensin Converting Enzyme in Smooth Muscle and Endothelial Cells
Lucero, H.A., Kintsurashvili,, E., Marketou, M.E. and Gavras, H.
J: Biol. Chem., ***285(8)***, *5555-5568 (2010)*

The angiotensin converting enzyme (ACE) catalyzes the extracellular formation of angiotensin II, and degradation of bradykinin, thus regulating blood pressure and renal handling of electrolytes. We have previously shown that exogenously added ACE elicited transcriptional regulation independent of its enzymatic activity. Because transcriptional regulation generates from protein-DNA interactions within the cell nucleus we have investigated the initial cellular response to exogenous ACE and the putative internalization of the enzyme in smooth muscle cells (SMC) and endothelial cells (EC). The following phenomena were observed when ACE was added to cells in culture: 1) it bound to SMC and EC with high affinity (K_d = 361.5 ± 60.5 pM) and with a low binding occupancy (B_{max} = 335.0 ± 14.0 molecules/cell); 2) it triggered cellular signaling resulting in late activation of focal adhesion kinase and SHP2; 3) it modulated platelet-derived growth factor receptor-β signaling; 4) it was endocytosed by SMC and EC; and 5) it transited through the early endosome, partially occupied the late endosome and the lysosome, and was localized to the nuclei. The incorporation of ACE or a fragment of it into the nuclei reached saturation at 120 min, and was preceded by a lag time of 40 min. Internalized ACE was partially cleaved into small fragments. These results revealed that extracellular ACE modulated cell signaling properties, and that

SMC and EC have a pathway for delivery of extracellular ACE to the nucleus, most likely involving cell surface receptor(s) and requiring transit through late endosome/lysosome compartments.

3.1424 Green tea catechin EGCG inhibits ileal apical sodium bile acid transporter ASBT
Annaba, F., Kumar, P., Dudeja, A.K., Saksena, S., Gill, R.K. and Alrefai, W.A.
AM. J. Gastrointest. Liver Physiol., ***298***, *G467-G473 (2010)*

Green tea catechins exhibit hypocholesterolemic effects probably via their inhibitory effects on intestinal bile acid absorption. Ileal apical sodium-dependent bile acid transporter (ASBT) is responsible for reabsorption of bile acids. The present studies were, therefore, designed to investigate the modulation of ASBT function and membrane expression by green tea catechins in human embryonic kidney HEK-293 cells stably transfected with ASBT-V5 fusion protein and intestinal Caco-2 monolayers. Our data showed that ASBT activity was significantly decreased by (–)-epigallocatechin-3-gallate (EGCG) but not other green tea catechins. Inhibition of PKC, phosphatidylinositol 3-kinase, and MAPK-dependent pathways failed to block the reduction in ASBT activity by EGCG. Kinetics studies showed a significant decrease in the V_{max} of the transporter, whereas total ASBT content on the plasma membrane was unaltered by EGCG. Concomitant with the decrease in ASBT function, EGCG significantly reduced ASBT pool in the detergent-insoluble fraction, while increasing its presence in the detergent-soluble fraction of plasma membrane. Furthermore, EGCG decreased the association of ASBT with floating lipid raft fractions of cellular membrane on **Optiprep** density gradient. In conclusion, our data demonstrate a novel role of lipid rafts in the modulation of ASBT function by the dietary component EGCG, which may underlie the hypocholesterolemic effects of green tea.

3.1425 Rituximab inhibits B-cell receptor signaling
Kheirallah, S., Caron, P., Gross, E., Quillet-Mary, A., Bertrand-Michel, J., Fournie, J-J., Laurent, G. and Bezombes, C.
Blood, ***115(5)***, *985-994 (2010)*

Rituximab (RTX), a monoclonal antibody directed against the CD20 protein, is a drug commonly used in the treatment of B-cell–derived lymphoid neoplasias and of antibody-mediated autoimmune diseases. In addition to cell- and complement-mediated B-cell depletion, RTX is thought to inhibit B-cell survival and proliferation through negative regulation of canonical signaling pathways involving Akt, ERK, and mammalian target of rapamycin. However, surprisingly, although B-cell receptor (BCR) signaling has been considered critical for normal and more recently, for neoplastic B cells, the hypothesis that RTX could target BCR has never been investigated. Using follicular lymphoma cell lines as models, as well as normal B cells, we show here, for the first time, that pretreatment with RTX results in a time-dependent inhibition of the BCR-signaling cascade involving Lyn, Syk, PLCγ2, Akt, and ERK, and calcium mobilization. The inhibitory effect of RTX correlates with decrease of raft-associated cholesterol, complete inhibition of BCR relocalization into lipid raft microdomains, and down-regulation of BCR immunoglobulin expression. Thus, RTX-mediated alteration of BCR expression, dynamics, and signaling might contribute to the immunosuppressive activity of the drug.

3.1426 Detergent-resistant microdomains mediate activation of host cell signaling in response to attaching–effacing bacteria
Shen-Tu, G., Schauer, D.B., Jones, N.L. and Sherman, P.M.
Lab. Invest., ***90(2)***, *266-281 (2010)*

Enterohemorrhagic Escherichia coli (EHEC) O157:H7 causes outbreaks of bloody diarrhea and the hemolytic–uremic syndrome. EHEC intimately adheres to epithelial cells, effaces microvilli and induces attaching–effacing (AE) lesions. Detergent-resistant microdomains (lipid rafts) serve as membrane platforms for the recruitment of signaling complexes to mediate host responses to infection. The aim of this study was to define the role of lipid rafts in activating signal transduction pathways in response to AE bacterial pathogens. Epithelial cell monolayers were infected with EHEC (MOI 100:1, 3 h, 37°C) and lipid rafts isolated by buoyant density ultracentrifugation. Phosphoinositide 3-kinase (PI3K) localization to lipid rafts was confirmed using PI3K and anti-caveolin-1 antibodies. Mice with cholesterol storage disease Niemann–Pick, type C were used as in vivo models to confirm the role of lipid rafts in mediating signaling response to AE organisms. In contrast to uninfected cells, PI3K was recruited to lipid rafts in response to EHEC infection. Metabolically active bacteria and cells with intact cholesterol-rich microdomains were necessary for the recruitment of second messengers to lipid rafts. Recruitment of PI3K to lipid rafts was independent of the intimin (eaeA) gene, type III secretion system, and production of Shiga-like toxins.

Colonization of NPC−/− colonic mucosa by Citrobacter rodentium and AE lesion formation were both delayed, compared with wild-type mice infected with the murine-specific AE bacterial pathogen. C. rodentium-infected NPC−/− mice had reduced colonic epithelial hyperplasia (64±8.251 vs 112±2.958 μm; $P<0.05$) and decreased secretion of IFN-γ (17.6±17.6 vs 71±26.3 pg/ml, $P<0.001$). Lipid rafts mediate host cell signal transduction responses to AE bacterial infections both in vitro and in vivo. These findings advance the current understanding of microbial–eukaryotic cell interactions in response to enteric pathogens that hijack signaling responses mediated through lipid rafts.

3.1427 Molecular Species of Phosphatidylinositol-Cycle Intermediates in the Endoplasmic Reticulum and Plasma Membrane

Shulga, Y.V., Mmyers, D.S., Ivanova, P.T., Milne, S.B., Brown, H.A., Topham, M.K. and Epand, R.M.
*Biochemistry, **49(2)**, 312-317 (2010)*

Phosphatidylinositol (PI) turnover is a process requiring both the plasma and ER membranes. We have determined the distribution of phosphatidic acid (PA) and PI and their acyl chain compositions in these two subcellular membranes using mass spectrometry. We assessed the role of PI cycling in determining the molecular species and quantity of these lipids by comparing the compositions of the two membranes isolated from embryonic fibroblasts obtained from diacylglycerol kinase ε (DGKε) knockout (KO) and wild-type (WT) mice. In the KO cells, the conversion of arachidonoyl-rich DAG to PA is blocked by the absence of DGKε, resulting in a reduction in the rate of PI cycling. The acyl chain composition is very similar for PI and PA in the endoplasmic reticulum (ER) versus plasma membrane (PM) and for WT versus KO. However, the acyl chain profile for PI is very different from that for PA. This indicates that DGKε is not facilitating the direct transfer of a specific species of PA between the PM and the ER. Approximately 20% of the PA in the ER membrane has one short acyl chain of 14 or fewer carbons. These species of PA are not converted into PI but may play a role in stabilizing regions of high positive curvature in the ER. There are also PI species in both the ER and PM for which there is no detectable PA precursor, indicating that these species of PI are unlikely to arise via the PI cycle. We find that in the PM of KO cells the levels of PI and of PA are decreased ~3-fold in comparison with those in either the PM of WT cells or the ER of KO cells. The PI cycle is slowed in the KO cells; hence, the lipid intermediates of the PI cycle can no longer be interconverted and are depleted from the PI cycle by conversion to other species. There is less of an effect of the depletion in the ER where de novo synthesis of PA occurs in comparison with the PM.

3.1428 Sphingomyelin-rich domains are sites of lysenin oligomerization: Implications for raft studies

Kulma, M., herec, M., Grudzinski, W., Anderluh, G., Gruszecki, W., Kwiarkowska, K. and Sobota, A.
*Biochim. Biophys. Acta, **1798**, 471-481 (2010)*

Lysenin is a self-assembling, pore-forming toxin which specifically recognizes sphingomyelin. Mutation of tryptophan 20 abolishes lysenin oligomerization and cytolytic activity. We studied the interaction of lysenin WT and W20A with sphingomyelin in membranes of various lipid compositions which, according to atomic force microscopy studies, generated either homo- or heterogeneous sphingomyelin distribution. Liposomes composed of SM/DOPC, SM/DOPC/cholesterol and SM/DPPC/cholesterol could bind the highest amounts of GST-lysenin WT, as shown by surface plasmon resonance analysis. These lipid compositions enhanced the release of carboxyfluorescein from liposomes induced by lysenin WT, pointing to the importance of heterogeneous sphingomyelin distribution for lysenin WT binding and oligomerization. Lysenin W20A bound more weakly to sphingomyelin-containing liposomes than did lysenin WT. The same amounts of lysenin W20A bound to sphingomyelin mixed with either DOPC or DPPC, indicating that the binding was not affected by sphingomyelin distribution in the membranes. The mutant lysenin had a limited ability to penetrate hydrophobic region of the membrane as indicated by measurements of surface pressure changes. When applied to detect sphingomyelin on the cell surface, lysenin W20A formed large conglomerates on the membrane, different from small and regular clusters of lysenin WT. Only lysenin WT recognized sphingomyelin pool affected by formation of raft-based signaling platforms. During fractionation of Triton X-100 cell lysates, SDS-resistant oligomers of lysenin WT associated with membrane fragments insoluble in Triton X-100 while monomers of lysenin W20A partitioned to Triton X-100-soluble membrane fractions. Altogether, the data suggest that oligomerization of lysenin WT is a prerequisite for its docking in raft-related domains.

3.1429 PPARγ Ligand 15-Deoxy-delta 12,14-Prostaglandin J2 Sensitizes Human Colon Carcinoma Cells to TWEAK-induced Apoptosis

Dionne, S., Levy, E., Levesque, D. and Seidman, E.G.

*Anticancer Res., **30**, 157-166 (2010)*

Background: Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) has been shown to induce colon cancer cell apoptosis in the presence of interferon-γ. We hypothesized that co-treatment using TWEAK with other pro-apoptosis agents could sensitize death receptor-resistant colon cancer cells. Materials and Methods: The effects of chemopreventive agents and TWEAK on cell death and apoptosis were determined using propidium iodide (PI) exclusion and M30 CytoDEATH. Results: We found that 15d-PGJ_2 sensitizes colon cancer cells to TWEAK-induced apoptosis. Caspase inhibition reduced 15d-PGJ_2-, but not 15d-PGJ_2+TWEAK-induced apoptosis. 15d-PGJ_2 promoted reactive oxygen species (ROS) production and dissipation of mitochondrial potential ($\Delta\Psi_m$) that were more marked with combined treatment. ROS, $\Delta\Psi_m$ and cell death were partially normalized by the antioxidant N-acetylcysteine. TWEAK induced nuclear factor-kappa B activation, which was attenuated by 15d-PGJ_2. 15d-PGJ_2 reduced the expression of the anti-apoptotic proteins BCL-X_L and MCL-1, while increasing BAX and translocation of cytochrome c and apoptosis-inducing factor. Conclusion: 15d-PGJ_2 sensitized cancer cells to TWEAK-induced apoptosis through an ROS-dependent cell death pathway and may have chemotherapeutic utility as an apoptosis-enhancing agent.

3.1430 The Matrix Peptide Exporter HAF-1 Signals a Mitochondrial UPR by Activating the Transcription Factor ZC376.7 in C. elegans
Haynes, C.M., Yang, Y., Blais, S.P., Neubert, T.A. and Ron, D.
*Mol. Cell., **37(4)**, 529-540 (2010)*

Genetic analyses previously implicated the matrix-localized protease ClpP in signaling the stress of protein misfolding in the mitochondrial matrix to activate nuclear-encoded mitochondrial chaperone genes in *C. elegans* (UPR^{mt}). Here, we report that *haf-1*, a gene encoding a mitochondria-localized ATP-binding cassette protein, is required for signaling within the UPR^{mt} and for coping with misfolded protein stress. Peptide efflux from isolated mitochondria was ATP dependent and required HAF-1 and the protease ClpP. Defective UPR^{mt} signaling in the *haf-1*-deleted worms was associated with failure of the bZIP protein, ZC376.7, to localize to nuclei in worms with perturbed mitochondrial protein folding, whereas *zc376.7*(RNAi) strongly inhibited the UPR^{mt}. These observations suggest a simple model whereby perturbation of the protein-folding environment in the mitochondrial matrix promotes ClpP-mediated generation of peptides whose *haf-1*-dependent export from the matrix contributes to UPR^{mt} signaling across the mitochondrial inner membrane.

3.1431 R-Ras regulates β1-integrin trafficking via effects on membrane ruffling and endocytosis
Conklin, M.C., Ada-Nguema, A., Parsons, M., Riching, K.M. and Keely, P.J.
*BMC Cell Biology, **11**, 14-28 (2010)*

Background
Integrin-mediated cell adhesion and spreading is dramatically enhanced by activation of the small GTPase, R-Ras. Moreover, R-Ras localizes to the leading edge of migrating cells, and regulates membrane protrusion. The exact mechanisms by which R-Ras regulates integrin function are not fully known. Nor is much known about the spatiotemporal relationship between these two molecules, an understanding of which may provide insight into R-Ras regulation of integrins.
Results
GFP-R-Ras localized to the plasma membrane, most specifically in membrane ruffles, in Cos-7 cells. GFP-R-Ras was endocytosed from these ruffles, and trafficked via multiple pathways, one of which involved large, acidic vesicles that were positive for Rab11. Cells transfected with a dominant negative form of GFP-R-Ras did not form ruffles, had decreased cell spreading, and contained numerous, non-trafficking small vesicles. Conversely, cells transfected with the constitutively active form of GFP-R-Ras contained a greater number of ruffles and large vesicles compared to wild-type transfected cells. Ruffle formation was inhibited by knock-down of endogenous R-Ras with siRNA, suggesting that activated R-Ras is not just a component of, but also an architect of ruffle formation. Importantly, β_1-integrin co-localized with endogenous R-Ras in ruffles and endocytosed vesicles. Expression of dominant negative R-Ras or knock down of R-Ras by siRNA prevented integrin accumulation into ruffles, impaired endocytosis of β_1-integrin, and decreased β_1-integrin-mediated adhesion. Knock-down of R-Ras also perturbed the dynamics of another membrane-localized protein, GFP-VSVG, suggesting a more global role for R-Ras on membrane dynamics. However, while R-Ras co-internalized with integrins, it did not traffic with VSVG, which instead moved laterally out of ruffles within the plane of the membrane, suggesting multiple levels of regulation of and by R-Ras.

Conclusions
Our results suggest that integrin function involves integrin trafficking via a cycle of membrane protrusion, ruffling, and endocytosis regulated by R-Ras, providing a novel mechanism by which integrins are linked to R-Ras through control of membrane dynamics.

3.1432 Acquisition of Complement Resistance through Incorporation of CD55/Decay-Accelerating Factor into Viral Particles Bearing Baculovirus GP64
Kaname, Y., Tani, H., Kataoka, C., Shiokawa, M., Taguwa, S., Abe, T., Moriishi, K., Kinoshita, T. and Matsuura, Y.
J. Virol., ***84(7)***, *3210-3219 (2010)*

A major obstacle to gene transduction by viral vectors is inactivation by human complement in vivo. One way to overcome this is to incorporate complement regulatory proteins, such as CD55/decay accelerating factor (DAF), into viral particles. Lentivirus vectors pseudotyped with the baculovirus envelope protein GP64 have been shown to acquire more potent resistance to serum inactivation and longer transgene expression than those pseudotyped with the vesicular stomatitis virus (VSV) envelope protein G. However, the molecular mechanisms underlying resistance to serum inactivation in pseudotype particles bearing the GP64 have not been precisely elucidated. In this study, we generated pseudotype and recombinant VSVs bearing the GP64. Recombinant VSVs generated in human cell lines exhibited the incorporation of human DAF in viral particles and were resistant to serum inactivation, whereas those generated in insect cells exhibited no incorporation of human DAF and were sensitive to complement inactivation. The GP64 and human DAF were detected on the detergent-resistant membrane and were coprecipitated by immunoprecipitation analysis. A pseudotype VSV bearing GP64 produced in human DAF knockdown cells reduced resistance to serum inactivation. In contrast, recombinant baculoviruses generated in insect cells expressing human DAF or carrying the human DAF gene exhibited resistance to complement inactivation. These results suggest that the incorporation of human DAF into viral particles by interacting with baculovirus GP64 is involved in the acquisition of resistance to serum inactivation.

3.1433 Endocytosis of the Anthrax Toxin Is Mediated by Clathrin, Actin and Unconventional Adaptors
Abrami, L., Bischofberger, M., Kubnz, B., Groux, R. and van der Goot, F.G.
PloSPathogens, ***6(3)***, *e1000792 (2010)*

The anthrax toxin is a tripartite toxin, where the two enzymatic subunits require the third subunit, the protective antigen (PA), to interact with cells and be escorted to their cytoplasmic targets. PA binds to cells via one of two receptors, TEM8 and CMG2. Interestingly, the toxin times and triggers its own endocytosis, in particular through the heptamerization of PA. Here we show that PA triggers the ubiquitination of its receptors in a β-arrestin-dependent manner and that this step is required for clathrin-mediated endocytosis. In addition, we find that endocytosis is dependent on the heterotetrameric adaptor AP-1 but not the more conventional AP-2. Finally, we show that endocytosis of PA is strongly dependent on actin. Unexpectedly, actin was also found to be essential for efficient heptamerization of PA, but only when bound to one of its 2 receptors, TEM8, due to the active organization of TEM8 into actin-dependent domains. Endocytic pathways are highly modular systems. Here we identify some of the key players that allow efficient heptamerization of PA and subsequent ubiquitin-dependent, clathrin-mediated endocytosis of the anthrax toxin.

3.1434 Redox signaling via lipid raft clustering in homocysteine-induced injury of podocytes
Zhang, C., Hu, J-J., Xia, M., Boini, K.M., Brimson, C. and Li, P-L.
Biochim. Biophys. Acta, ***1803***, *482-491 (2010)*

Our recent studies have indicated that hyperhomocysteinemia (hHcys) may induce podocyte damage, resulting in glomerulosclerosis. However, the molecular mechanisms mediating hHcys-induced podocyte injury are still poorly understood. In the present study, we first demonstrated that an intact NADPH oxidase system is present in podocytes as shown by detection of its membrane subunit ($gp91^{phox}$) and cytosolic subunit ($p47^{phox}$). Then, confocal microscopy showed that $gp91^{phox}$ and $p47^{phox}$ could be aggregated in lipid raft (LR) clusters in podocytes treated with homocysteine (Hcys), which were illustrated by their colocalization with cholera toxin B, a common LR marker. Different mechanistic LR disruptors, either methyl-β-cyclodextrin (MCD) or filipin abolished such Hcys-induced formation of LR-$gp91^{phox}$ or LR-$p47^{phox}$ transmembrane signaling complexes. By flotation of detergent-resistant membrane fractions we found that $gp91^{phox}$ and $p47^{phox}$ were enriched in LR fractions upon Hcys stimulation, and such enrichment of NADPH oxidase subunits and increase in its enzyme activity were blocked by MCD or

filipin. Functionally, disruption of LR clustering significantly attenuated Hcys-induced podocyte injury, as shown by their inhibitory effects on Hcys-decreased expression of slit diaphragm molecules such as nephrin and podocin. Similarly, Hcys-increased expression of desmin was also reduced by disruption of LR clustering. In addition, inhibition of such LR-associated redox signaling prevented cytoskeleton disarrangement and apoptosis induced by Hcys. It is concluded that NADPH oxidase subunits aggregation and consequent activation of this enzyme through LR clustering is an important molecular mechanism triggering oxidative injury of podocytes induced by Hcys.

3.1435 Low Density Subcellular Fractions Enhance Disease-specific Prion Protein Misfolding
Graham, J.F., Agarwal, S., Kurian, D., Kirby, L., Pinheiro, T.J. and gill, A.C.
J. Biol. Chem., ***285(13)***, *9868-9880 (2010)*

The production of prion particles *in vitro* by amplification with or without exogenous seed typically results in infectivity titers less than those associated with PrP^{Sc} isolated *ex vivo* and highlights the potential role of co-factors that can catalyze disease-specific prion protein misfolding *in vivo*. We used a cell-free conversion assay previously shown to replicate many aspects of transmissible spongiform encephalopathy disease to investigate the cellular location of disease-specific co-factors using fractions derived from gradient centrifugation of a scrapie-susceptible cell line. Fractions from the low density region of the gradient doubled the efficiency of conversion of recombinant PrP. These fractions contain plasma membrane and cytoplasmic proteins, and conversion enhancement can be achieved using PrP^{Sc} derived from two different strains of mouse-passaged scrapie as seed. Equivalent fractions from a second scrapie-susceptible cell line also stimulate conversion. We also show that subcellular fractions enhancing disease-specific prion protein conversion prevent *in vitro* fibrillization of recombinant prion protein, suggesting the existence of separate, competing mechanisms of disease-specific and nonspecific misfolding *in vivo*.

3.1436 Cholesterol–Protein Interaction: Methods and Cholesterol Reporter Molecules
Gimpl, G.
Subcellular Biochem., ***51***, *1-45 (2010)*

Cholesterol is a major constituent of the plasma membrane in eukaryotic cells. It regulates the physical state of the phospholipid bilayer and is crucially involved in the formation of membrane microdomains. Cholesterol also affects the activity of several membrane proteins, and is the precursor for steroid hormones and bile acids. Here, methods are described that are used to explore the binding and/or interaction of proteins to cholesterol. For this purpose, a variety of cholesterol probes bearing radio-, spin-, photoaffinity- or fluorescent labels are currently available. Examples of proven cholesterol binding molecules are polyene compounds, cholesterol-dependent cytolysins, enzymes accepting cholesterol as substrate, and proteins with cholesterol binding motifs. Main topics of this report are the localization of candidate membrane proteins in cholesterol-rich microdomains, the issue of specificity of cholesterol–protein interactions, and applications of the various cholesterol probes for these studies.

3.1437 Vimentin-mediated signalling is required for IbeA+ *E. coli* K1 invasion of human brain microvascular endothelial cells
Chi, F., Jong, T.D., Wang, L., Ouyang, Y., Wu, C., Li, W. and Huang, S-H.
Biochem. J., ***427***, *79-90 (2010)*

IbeA in meningitic *Escherichia coli* K1 strains has been described previously for its role in invasion of BMECs (brain microvascular endothelial cells). Vimentin was identified as an IbeA-binding protein on the surface of HBMECs (human BMECs). In the present study, we demonstrated that vimentin is a primary receptor required for IbeA+ *E. coli* K1-induced signalling and invasion of HBMECs, on the basis of the following observations. First, E44 (IbeA+ *E. coli* K1 strain) invasion was blocked by vimentin inhibitors (withaferin A and acrylamide), a recombinant protein containing the vimentin head domain and an antibody against the head domain respectively. Secondly, overexpression of GFP (green fluorescent protein)–vimentin and GFP–VDM (vimentin head domain deletion mutant) significantly increased and decreased bacterial invasion respectively. Thirdly, bacterial invasion was positively correlated with phosphorylation of vimentin at Ser^{82} by CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) and IbeA+ *E. coli*-induced phosphorylation of ERK (extracellular-signal-regulated kinase). Blockage of CaMKII by KN93 and inhibition of ERK1/2 phosphorylation by PD098059 resulted in reduced IbeA+ *E. coli* invasion. Fourthly, IbeA+ *E. coli* and IbeA-coated beads induced the clustering of vimentin that was correlated with increased entry of bacteria and beads. Lastly, IbeA+ *E. coli* K1 invasion was inhibited by lipid-raft-disrupting agents (filipin and nystatin) and caveolin-1 siRNA (small interfering RNA), suggesting that

caveolae/lipid rafts are signalling platforms for inducing IbeA–vimentin-mediated *E. coli* invasion of HBMECs. Taken together, the present studies suggest that a dynamic and function-related interaction between IbeA and its primary receptor vimentin at HBMEC membrane rafts leads to vimentin phosphorylation and ERK-mediated signalling, which modulate meningitic *E. coli* K1 invasion.

3.1438 Mechanisms of proximal tubule sodium transport regulation that link extracellular fluid volume and blood pressure

McDonough, A.A.

*Am. J. Physiol. Regyl. Integr. Comp. Physiol., **298**, R851-R861 (2010)*

One-hundred years ago, Starling articulated the interdependence of renal control of circulating blood volume and effective cardiac performance. During the past 25 years, the molecular mechanisms responsible for the interdependence of blood pressure (BP), extracellular fluid volume (ECFV), the renin-angiotensin system (RAS), and sympathetic nervous system (SNS) have begun to be revealed. These variables all converge on regulation of renal proximal tubule (PT) sodium transport. The PT reabsorbs two-thirds of the filtered Na^+ and volume at baseline. This fraction is decreased when BP or perfusion pressure is increased, during a high-salt diet (elevated ECFV), and during inhibition of the production of ANG II; conversely, this fraction is increased by ANG II, SNS activation, and a low-salt diet. These variables all regulate the distribution of the Na^+/H^+ exchanger isoform 3 (NHE3) and the Na^+-phosphate cotransporter (NaPi2), along the apical microvilli of the PT. Natriuretic stimuli provoke the dynamic redistribution of these transporters along with associated regulators, molecular motors, and cytoskeleton-associated proteins to the base of the microvilli. The lipid raft-associated NHE3 remains at the base, and the nonraft-associated NaPi2 is endocytosed, culminating in decreased Na^+ transport and increased PT flow rate. Antinatriuretic stimuli return the same transporters and regulators to the body of the microvilli associated with an increase in transport activity and decrease in PT flow rate. In summary, ECFV and BP homeostasis are, at least in part, maintained by continuous and acute redistribution of transporter complexes up and down the PT microvilli, which affect regulation of PT sodium reabsorption in response to fluctuations in ECFV, BP, SNS, and RAS.

3.1439 Isoflurane via TGF-β1 release increases caveolae formation and organizes sphingosine kinase signaling in renal proximal tubules

Song, J.H., Kim, M., Park, S.W., Chen, S.W.C., Pitson, S.M. and Lee, H.T.

*Am. J. Physiol. Renal Physiol., **298**, F1041-F1050 (2010)*

We previously showed that the inhalational anesthetic isoflurane protects against renal proximal tubule necrosis via isoflurane-mediated stimulation and translocation of sphingosine kinase-1 (SK1) with subsequent synthesis of sphingosine-1-phosphate (S1P) in renal proximal tubule cells (Kim M, Kim M, Kim N, D'Agati VD, Emala CW Sr, Lee HT. *Am J Physiol Renal Physiol* 293: F1827–F1835, 2007). We also demonstrated that the anti-necrotic and anti-inflammatory effect of isoflurane is due in part to phosphatidylserine (PS) externalization and subsequent release of transforming growth factor-β1 (TGF-β1) (Lee HT, Kim M, Kim J, Kim N, Emala CW. *Am J Nephrol* 27: 416–424, 2007). In this study, we tested the hypothesis that isoflurane, via TGF-β1 release, increases caveolae formation in the buoyant fraction of the cell membrane of human renal proximal tubule (HK-2) cells to organize SK1 and S1P signaling. To detect SK1 protein in the caveolae/caveolin fractions, we overexpressed human SK1 in HK-2 cells (SK1-HK-2). SK1-HK-2 cells exposed to isoflurane increased caveolae/caveolin formation in the buoyant membrane fractions which contained key signaling intermediates involved in isoflurane-mediated renal tubule protection, including S1P, SK1, ERK MAPK, and TGF-β1 receptors. Furthermore, treating SK1-HK-2 cells with recombinant TGF-β1 or PS liposome mixture increased caveolae formation, mimicking the effects of isoflurane. Conversely, TGF-β1-neutralizing antibody blocked the increase in caveolae formation induced by isoflurane in SK1-HK-2 cells. The increase in SK1 activity in the caveolae-enriched fractions from isoflurane-treated nonlentivirus-infected HK-2 cells, while smaller in magnitude, was qualitatively similar to that found in the SK1-HK-2 cell line. Finally, isoflurane also increased caveolae formation in the kidneys of TGF-β1 +/+ mice but not in TGF-β1 +/– mice (mice with reduced levels of TGF-β1). Our study demonstrates that isoflurane organizes several key cytoprotective signaling intermediates including TGF-β1 receptors, SK1 and ERK, within the caveolae fraction of the plasma membrane. Our findings may help to unravel the cellular signaling pathways of volatile anesthetic-mediated renal protection and lead to new therapeutic applications of inhalational anesthetics during the perioperative period.

3.1440 Control of Rhodopsin's Active Lifetime by Arrestin-1 Expression in Mammalian Rods

Gross, O.P. and Burns, M.E.

J. Neurosci., ***30(9)***, *3450-3457 (2010)*

In rod photoreceptors, deactivation of the light-activated G-protein-coupled receptor rhodopsin (R*) is initiated by phosphorylation and completed through subsequent binding of visual arrestin (Arr1). The *in vivo* kinetics of these individual interactions have proven difficult to determine with precision since R* lifetime is much shorter than the lifetimes of downstream G-protein and effector molecules. Here, we have used a transgenic mouse line with accelerated downstream deactivation kinetics to reveal the contribution of Arr1 binding to the overall time course of rhodopsin deactivation. Photoresponses revealed that the lifetime of R* is significantly increased in rods that express half of the normal amount of Arr1, in a manner consistent with a twofold decrease in the rate of Arr1 binding across a wide range of flash strengths. A basic model of photoresponse deactivation consistent with established photoreceptor biochemistry shows that R* phosphorylation and Arr1 binding occur with a time constant of ~40 ms in wild-type mouse rods, much faster than previous estimates.

3.1441 **Troubleshooting methods for APP processing *in vitro***
Sastre, M.
J. Pharmacol. Toxicol methods, ***61***, *86-91 (2010)*

Introduction
The amyloid hypothesis states that Aβ is the main trigger for Alzheimer's disease. This report is focused in the study of the processing of the Amyloid Precursor Protein (APP) as a procedure to investigate the molecular mechanisms that may result in changes in the levels of Aβ.
Methods
Here we analyse different methodologies for Aβ determination, soluble APP, APP-Carboxy terminus fragments (CTFs) and enzymes for synthesis (secretases) and degradation of Aβ. In addition the advantages and disadvantages of different methodologies are discussed.
Discussion
The potential value of these procedures is described in the context of the function of APP and the different fragments derived from its cleavage.

3.1442 **The Human Polyoma JC Virus Agnoprotein Acts as a Viroporin**
Suzuki, T., Orba, Y., Okada, Y., Sunden, Y., Kimura, T., Tanaka, S., Nagashima, K., Hall, W.W., Sawa, H.
PloSPathogens, ***6(3)***, *e1000801 (2010)*

Virus infections can result in a range of cellular injuries and commonly this involves both the plasma and intracellular membranes, resulting in enhanced permeability. Viroporins are a group of proteins that interact with plasma membranes modifying permeability and can promote the release of viral particles. While these proteins are not essential for virus replication, their activity certainly promotes virus growth. Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease resulting from lytic infection of oligodendrocytes by the polyomavirus JC virus (JCV). The genome of JCV encodes six major proteins including a small auxiliary protein known as agnoprotein. Studies on other polyomavirus agnoproteins have suggested that the protein may contribute to viral propagation at various stages in the replication cycle, including transcription, translation, processing of late viral proteins, assembly of virions, and viral propagation. Previous studies from our and other laboratories have indicated that JCV agnoprotein plays an important, although as yet incompletely understood role in the propagation of JCV. Here, we demonstrate that agnoprotein possesses properties commonly associated with viroporins. Our findings demonstrate that: (i) A deletion mutant of agnoprotein is defective in virion release and viral propagation; (ii) Agnoprotein localizes to the ER early in infection, but is also found at the plasma membrane late in infection; (iii) Agnoprotein is an integral membrane protein and forms homo-oligomers; (iv) Agnoprotein enhances permeability of cells to the translation inhibitor hygromycin B; (v) Agnoprotein induces the influx of extracellular Ca^{2+}; (vi) The basic residues at amino acid positions 8 and 9 of agnoprotein key are determinants of the viroporin activity. The viroporin-like properties of agnoprotein result in increased membrane permeability and alterations in intracellular Ca^{2+} homeostasis leading to membrane dysfunction and enhancement of virus release.

3.1443 **Mapping of Vps21 and HOPS Binding Sites in Vps8 and Effect of Binding Site Mutants on Endocytic Trafficking**
Pawelec, A., Arsic, J. and Krölling, R.

*Eukaryot. Cell, **9(4)**, 602-610 (2010)*

Vps8 is a subunit of the CORVET tethering complex, which is involved in early-to-late endosome fusion. Here, we examine the role of Vps8 in membrane fusion at late endosomes in Saccharomyces cerevisiae. We demonstrate that Vps8 associates with membranes and that this association is independent of the class C/HOPS core complex and, contrary to a previous report, also independent of the Rab GTPase Vps21. Our data indicate that Vps8 makes multiple contacts with membranes. One of these membrane binding regions could be mapped to the N-terminal part of the protein. By two-hybrid analysis, we obtained evidence for a physical interaction between Vps8 and the Rab5 homologue Vps21. In addition, the interaction with the HOPS core complex was confirmed by immunoprecipitation experiments. By deletion analysis, the Vps21 and HOPS binding sites were mapped in Vps8. Deletions that abrogated HOPS core complex binding had a strong effect on the turnover of the endocytic cargo protein Ste6 and on vacuolar sorting of carboxypeptidase Y. In contrast, deletions that abolished Vps21 binding showed only a modest effect. This suggests that the Vps21 interaction is not essential for endosomal trafficking but may be important for some other aspect of Vps8 function.

3.1444 Amot Recognizes a Juxtanuclear Endocytic Recycling Compartment via a Novel Lipid Binding Domain

Heller, B., Adu-Gyamfi, E., Smith-Kinnaman, W., Babbey, C., Vora, M., Xue, Y., Bittman, R., Stahelin, R.V. and Wells, C.D.
*J. Biol. Chem., **285(16)**, 12308-12320 (2010)*

Polarity proteins promote the asymmetric organization of cells by orienting intracellular sorting mechanisms, such as protein trafficking and cytoskeletal assembly. The localization of individual polarity proteins in turn is often determined by association with factors that mediate contact with other cells or the substratum. This arrangement for the Par and Crb apical polarity complexes at the tight junction is disrupted by the adaptor protein Amot. Amot directly binds the scaffolding proteins Patj and Mupp1 and redistributes them and their binding partners from the plasma membrane to endosomes. However, the mechanism by which Amot is targeted to endosomes is unknown. Here, a novel lipid binding domain within Amot is shown to selectively bind with high affinity to membranes containing monophosphorylated phosphatidylinositols and cholesterol. With similar lipid specificity, Amot inserts into and tubulates membranes *in vitro* and enlarges perinuclear endosomal compartments in cells. Based on the similar distribution of Amot with cholesterol, Rab11, and Arf6, such membrane interactions are identified at juxtanuclear endocytic recycling compartments. Taken together, these findings indicate that Amot is targeted along with associated apical polarity proteins to the endocytic recycling compartment via this novel membrane binding domain.

3.1445 A Role for the C Terminus of Mopeia Virus Nucleoprotein in Its Incorporation into Z Protein-Induced Virus-Like Particles

Shtanko, O., Imai, M., Goto, H., Lakashevich, I.S., Neumann, G., Watanabe, T. and Kawaoka, Y.
*J. Virol., **84(10)**, 5415-5422 (2010)*

Arenaviruses are enveloped, negative-strand RNA viruses. For several arenaviruses, virus-like particle (VLP) formation requires the viral matrix Z protein. However, the mechanism by which viral ribonucleoprotein complexes are incorporated into virions is poorly understood. Here, we show that the expression of the Z protein and nucleoprotein (NP) of Mopeia virus, a close relative of the pathogenic Lassa virus, resulted in the highly selective incorporation of the NP protein into Z protein-induced VLPs. Moreover, the Z protein promoted the association of NP with cellular membranes, suggesting that the association of NP, Z, and the cellular membranes may facilitate the efficient incorporation of NP into VLPs. By employing a series of NP deletion constructs and testing their VLP incorporation, we further demonstrated an important role for the C-terminal half of NP in its incorporation into VLPs.

3.1446 Myristoylated Naked2 Antagonizes Wnt-β-Catenin Activity by Degrading Dishevelled-1 at the Plasma Membrane

Hu, T., Li, C., Cao, Z., Van Raay, T.J., Smith, J.G., Willert, K., Solnica-Krezel, L. and Coffey, R.J.
*J. Biol. Chem., **285(18)**, 13561-13568 (2010)*

In *Drosophila*, *naked cuticle* is an inducible antagonist of the Wnt-β-catenin pathway, likely acting at the level of Dishevelled (Dsh/Dvl), an essential component of this pathway. The mechanism by which *naked*

cuticle and its two vertebrate orthologs, *Naked1* (*NKD1*) and *Naked2* (*NKD2*), inhibit Dvl function is unknown. NKD2 is myristoylated, a co-translational modification that leads to its plasma membrane localization. In contrast, myristoylation-deficient G2A NKD2 is cytoplasmic. Herein we show that the ability of Nkd2/NKD2 to antagonize Wnt-β-catenin activity during zebrafish embryonic development and in mammalian HEK293 cells is myristoylation-dependent. NKD2 and Dvl-1 interact and co-localize at the lateral membrane of polarized epithelial cells. In reciprocal overexpression and siRNA knockdown experiments, NKD2 and Dvl-1 destabilize each other via enhanced polyubiquitylation; this effect is also dependent upon Naked2 myristoylation. Cell fractionation and ubiquitylation assays indicate that endogenous NKD2 interacts with a slower migrating, ubiquitylated form of Dvl-1 in plasma membrane fractions. These results provide a mechanism by which NKD2 antagonizes Wnt signaling: myristoylated NKD2 interacts with Dvl-1 at the plasma membrane, and this interaction leads to their mutual ubiquitin-mediated proteasomal degradation.

3.1447 **Acidic calcium stores open for business: expanding the potential for intracellular Ca^{2+} signaling**
Patel, S. and Docompo, R.
*Trends in cell Biology, **20(5)**, 277-286 (2010)*

Changes in cytosolic calcium concentration are crucial for a variety of cellular processes in all cells. It has long been appreciated that calcium is stored and released from intracellular calcium stores such as the endoplasmic reticulum. However, emerging evidence indicates that calcium is also dynamically regulated by a seemingly disparate collection of acidic organelles. In this paper, we review the defining features of these 'acidic calcium stores' and highlight recent progress in understanding the mechanisms of uptake and release of calcium from these stores. We also examine the nature of calcium buffering within the stores, and summarize the physiological and pathophysiological significance of these ubiquitous organelles in calcium signaling.

3.1448 **Differential Pattern of Junctional Complex Lipid Raft Proteins in Human Conventional Outflow**
Mccarty, R.D., Beverley, R.M., Giovingo, M.C., Nolan, M.J., Yue, B.Y.J.T., Stamer, W.D. and Knepper, P.A.
*Invest. Ophthalmol. Vis. Sci., **51**, E-Abstract 3204 (2010)*

Purpose: The trabecular meshwork (TM) is a specialized tissue forming a biologic filter for aqueous humor. The two dominant cell types in the TM are Schlemm's canal (SC) endothelial cells on the inner wall of the collecting channels and TM cells that cover the TM beams. SC endothelial cells differ morphologically and functionally from TM endothelial cells. Exactly how these cells, their junctional complexes or their extracellular matrices, regulate aqueous outflow barrier resistance is poorly understood. The purpose of this study was to compare junctional complex lipid raft proteins of SC cells to that of TM cells.
Methods: Human SC and TM cells were grown to confluency in T-25 flasks. After two weeks at confluence, cells were lysed in ice cold lysis buffer containing 1% Triton X-100. Caveolin enriched lipid rafts were isolated using an **OptiPrep** density gradient (Sigma D1556). The preparation was centrifuged at 200,000 x g for 18 hrs; nine 1.0 ml fractions were pipetted from the top (lightest) to bottom (heaviest). Each fraction was analyzed for protein content. Equal amounts of proteins were resolved by SDS polyacrylamide gel electrophoresis and immunoblotted with a panel of junctional complex antibodies. Densitometry was performed.
Results: Profile of junctional complexes in enriched lipid rafts.

Conclusions: This is the first identification and comparison of the junctional complexes in SC and TM. Lipid raft proteins in SC cells are distinct from TM cells. SC cells express more annexin 2 than TM cells. Both have considerable amounts of occludin, zona occludens and other constituents of the lipid rafts. These differential expression patterns likely correspond to differences in function of TM and SC endothelial cells in modulating aqueous outflow resistance.

3.1449 **Differential Targeting of HSV Structural Proteins to Axons Requires Association of Viral Us9 Protein to Lipid Rafts**
LaVail, J.H., Huang, G., Cortez, D.A., Sucher, A. and Draper, J.M.
*Invest. Ophthalmol. Vis. Sci., **51**, E-abstract 3812 (2010)*

The Herpes simplex virus type 1 (HSV) envelope contains at least 10 glycoproteins, several of which are essential for viral spread leading to recurrent herpetic keratitis or viral encephalitis. How these glycoproteins are targeted within mature axons after synthesis is unclear.

Purpose: Our goal is to examine the axonal transport of the HSV envelope glycoproteins and to determine whether targeting of newly synthesized glycoproteins requires an association with the lipid rafts in membranes of host neurons. Further, we tested whether the viral protein, Us9, facilitates axonal transport of viral structural proteins, as well as that of viral capsids.
Methods: Murine retinas were pulse infected with *Us9*-null mutant or wild type (wt) HSV. After 5 days the optic pathways were dissected and prepared in an **Optiprep**® flotation assay to separate raft (detergent-resistant, DRM) and non-raft (detergent-soluble, DSM) membranes. The proteins were separated by PAGE and processed for Western blotting using antibodies to gB, gD, Us9, VP5 and synaptophysin (Syn). We used antisera to caveolin and transferrin and biotinolylated cholera toxin B subunit to GM1 as controls.
Results: By 5 days after infection with wt HSV, all proteins were transported to the optic tract. Two envelope glycoproteins (gB, gD), one capsid protein, VP5, and one membrane protein, Us9, were present in DRM fractions. In addition, gB, gD, Us9 and Syn, were found in DSM fractions. After infection with *Us9*-null HSV, the concentration of gB in both fractions was significantly reduced.
Conclusions: Lipid rafts play a role in the axonal transport of some, but not all of the HSV glycoproteins in axons of mature animals. Some glycoproteins are targeted independently of others. Specifically, the axonal transport of gB is impaired after *Us9*-null infection, but the transport of gD (and possibly gE and gC) is not impaired. Significantly, this impairment may account for the fact that cell-cell spread of new virus depends on gB expression. This suggests that axonal transport of HSV requires targeting of three subassemblies: membranes associated with gD, membranes associated with gB and the nucleocapsid.

3.1450 Soluble CD44 Increases Outflow Resistance in Porcine Organ CulturesSoluble CD44 Increases Outflow Resistance in Porcine Organ Cultures
Giovingo, M.C., McCarty, R.D., Beverley, R.M., Nolan, M.J., Samples, J.R., Yue, B.Y.J.T. and Knepper, P.A.
Invest. Ophthalmol. Vis. Sci., ***51***, *E-abstract 5838 (2010)*

Purpose:CD44 plays major roles in multiple physiological processes. The soluble CD44 (sCD44) concentration is significantly increased in the aqueous humor of primary open-angle glaucoma (POAG). Increased sCD44 in mouse eyes causes an increase in intraocular pressure (IOP). The purpose of this study was to determine if sCD44 changes outflow resistance in porcine anterior segment organ culture and profiles of lipid raft proteins of the trabecular meshwork (TM).
Methods:sCD44 was purified from human serum using anion exchange, hyaluronic acid (HA) affinity chromatography and immunoprecipitation. Anterior segments of porcine eyes were placed in organ culture and perfused with Dulbecco's modified eagle medium (DMEM). Flow rates were measured in eyes treated with sCD44, HA or DMEM. Perturbation of lipid raft containing proteins was assessed by **Optiprep** density gradient and Western blot analysis of dissected TM.
Results: Flow rates are expressed as percent change (+/-) from the baseline. Rates significantly decreased from baseline in HA and sCD44 infused eyes.
Analysis of lipid raft containing proteins of sCD44 infused porcine microdissected TMs and primary cultures of TM cells revealed a decrease in annexin 2 and an increase in caveolin-1 compared to that of controls.
Conclusions: Infusion of sCD44 significantly decreased outflow facility in porcine eyes. Notably, sCD44 caused a decrease in annexin 2 and an increase in caveolin-1 which may influence outflow resistance

3.1451 Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1
Boulter, E., Garcia-Mata, R., Guilluy, C., Dubash, A., Rossi, G., Brennwald, P.J. and Burridge, K.
Nature Cell Biol., ***12(5)***, *477-483 (2010)*

At steady state, most Rho GTPases are bound in the cytosol to Rho guanine nucleotide dissociation inhibitors (RhoGDIs)[1]. RhoGDIs have generally been considered to hold Rho proteins passively in an inactive state within the cytoplasm. Here we describe an evolutionarily conserved mechanism by which RhoGDI1 controls the homeostasis of Rho proteins in eukaryotic cells. We found that depletion of RhoGDI1 promotes misfolding and degradation of the cytosolic geranylgeranylated pool of Rho GTPases while activating the remaining membrane-bound fraction. Because RhoGDI1 levels are limiting, and Rho proteins compete for binding to RhoGDI1, overexpression of an exogenous Rho GTPase displaces endogenous Rho proteins bound to RhoGDI1, inducing their degradation and inactivation. These results raise important questions about the conclusions drawn from studies that manipulate Rho protein levels. In many cases the response observed may arise not simply from the overexpression itself but from additional effects on the levels and activity of other Rho GTPases as a result of competition for binding to RhoGDI1; this may require a re-evaluation of previously published studies that rely exclusively on these techniques.

3.1452 Osteopotentia regulates osteoblast maturation, bone formation, and skeletal integrity in mice
Sohaskey, M.L., Jiang, Y., Zhao, J.J., Mohr, A., Roemer, f. and Harland, R.M.
J. Cell Biol., ***189(3)***, *511-525 (2010)*

During skeletal development and regeneration, bone-forming osteoblasts respond to high metabolic demand by active expansion of their rough endoplasmic reticulum (rER) and increased synthesis of type I collagen, the predominant bone matrix protein. However, the molecular mechanisms that orchestrate this response are not well understood. We show that insertional mutagenesis of the previously uncharacterized *osteopotentia* (*Opt*) gene disrupts osteoblast function and causes catastrophic defects in postnatal skeletal development. *Opt* encodes a widely expressed rER-localized integral membrane protein containing a conserved SUN (Sad1/Unc-84 homology) domain. Mice lacking Opt develop acute onset skeletal defects that include impaired bone formation and spontaneous fractures. These defects result in part from a cell-autonomous failure of osteoblast maturation and a posttranscriptional decline in type I collagen synthesis, which is concordant with minimal rER expansion. By identifying Opt as a crucial regulator of bone formation in the mouse, our results uncover a novel rER-mediated control point in osteoblast function and implicate human *Opt* as a candidate gene for brittle bone disorders.

3.1453 Cholesterol loading in macrophages stimulates formation of ER-derived vesicles with elevated ACAT1 activity
Sakashita, N., Chang, C.C.Y., Lei, X., Fujiwara,, Y., Takeya, M.and Chang, T-Y.
J. Lipid Res., ***51***, *1263-1272 82010)*

ACAT1 is normally a resident enzyme in the endoplasmic reticulum (ER). We previously showed that treating macrophages with denatured LDL causes a large increase in ER-derived, ACAT1-positive vesicles. Here, we isolated ER membranes and ER-derived vesicles to examine their ACAT enzyme activity in vitro. The results showed that when macrophages are grown under normal conditions, ACAT1 is located in high density ER membrane; its enzymatic activity is relatively low. Loading macrophages with cholesterol did not increase the total cellular ACAT1 protein content significantly but caused more ACAT1 to appear in ER-derived vesicles. These vesicles exhibit lower density and are associated with markers of both ER and the trans-Golgi network. When normalized with equal ACAT1 protein mass, the enzymatic activities of ACAT1 in ER-derived vesicles were 3-fold higher than those present in ER membrane. Results using reconstituted ACAT enzyme assay showed that the increase in enzyme activity in ER-derived vesicles is not due to an increase in the cholesterol content associated with these vesicles. Overall, our results show that macrophages cope with cholesterol loading by using a novel mechanism: they produce more ER-derived vesicles with elevated ACAT1 enzyme activity without having to produce more ACAT1 protein.

3.1454 Biochemical Monitoring of the Early Endocytic Traffic of the Type I Interferon Receptor
Payelle-Brogard, B. and Pellegrini, S.
J. Interferon & Cytokine Res., ***30(2)***, *89-98 (2010)*

The type I interferon (IFN) receptor consists of two transmembrane chains IFNAR1 and IFNAR2, associated with the tyrosine kinases Tyk2 and Jak1, respectively. Binding of IFN to this receptor complex induces activation of Jak/Stat and non-Stat signaling pathways. Ligand binding also drives receptor internalization and sorting toward degradation or recycling. To gain insights into receptor trafficking and its relation to signaling, we performed subcellular organelle fractionation from IFN-stimulated Daudi cells and defined biochemically an early endosomal antigen-1 (EEA1)-positive compartment bearing the activated IFN receptor. Endosomes were thus purified by immunoaffinity isolation on anti-EEA1 antibodies-coated beads. The content of these purified endosomal fractions was analyzed by Western blot and proteomics. Shortly after IFN stimulation, robustly ubiquitinated IFNAR1 and a small amount of IFNAR2 were found in this endosomal compartment, which also contained tyrosine-phosphorylated Tyk2 and Jak1. These data strongly point to the prolonged interaction during traffic of the tyrosine kinases, still in an activated configuration, with the receptors. Among the major constituents of this EEA1-positive compartment, some proteins that have been implicated in IFN signaling were identified. Altogether, these observations suggest that trafficking of the IFN receptor through endosomes may regulate signaling pathways.

3.1455 Cellular factors implicated in prion replication

Abid, K., Morales, R. and Soto, C.
FEBS Lett., ***584***, *2409-2414 (2010)*

Prions are the unconventional infectious agents responsible for prion diseases, which are composed mainly by the misfolded prion protein (PrP^{Sc}) that replicates by converting the host associated cellular prion protein (PrP^{C}). Several lines of evidence suggest that other cellular components participate in prion conversion, however, the identity or even the chemical nature of such factors are entirely unknown. In this article we study the conversion factor activity by complementation of a PMCA procedure employing purified PrP^{C} and PrP^{Sc}. Our results show that the conversion factor is present in all major organs of diverse mammalian species, and is predominantly located in the lipid raft fraction of the cytoplasmic membrane. On the other hand, it is not present in the lower organisms tested (yeast, bacteria and flies). Surprisingly, treatments that eliminate the major classes of chemical molecules do not affect conversion activity, suggesting that various different compounds may act as conversion factor in vitro. This conclusion is further supported by experiments showing that addition of various classes of molecules have a small, but detectable effect on enhancing prion replication in vitro. More research is needed to elucidate the identity of these factors, their detailed mechanism of action and whether or not they are essential component of the infectious particle.

3.1456 A role for caveolin-1 in mechanotransduction of fetal type II epithelial cells

Wang, Y., Maciejewski, B.S., Drouillard, D., Santos, M., Hokenson, M.A., Hawwa, R.L., Huang, Z. and Sanchez-Esteban, J.
Am. J. Physiol. Lung Cell Mol. Physiol.,***298***, *L775-L783 (2010)*

Mechanical forces are critical for fetal lung development. Using surfactant protein C (SP-C) as a marker, we previously showed that stretch-induced fetal type II cell differentiation is mediated via the ERK pathway. Caveolin-1, a major component of the plasma membrane microdomains, is important as a signaling protein in blood vessels exposed to shear stress. Its potential role in mechanotransduction during fetal lung development is unknown. Caveolin-1 is a marker of type I epithelial cell phenotype. In this study, using immunocytochemistry, Western blotting, and immunogold electron microscopy, we first demonstrated the presence of caveolin-1 in embryonic *day 19* (E19) rat fetal type II epithelial cells. By detergent-free purification of lipid raft-rich membrane fractions and fluorescence immunocytochemistry, we found that mechanical stretch translocates caveolin-1 from the plasma membrane to the cytoplasm. Disruption of the lipid rafts with cholesterol-chelating agents further increased stretch-induced ERK activation and SP-C gene expression compared with stretch samples without disruptors. Similar results were obtained when caveolin-1 gene was knocked down by small interference RNA. In contrast, adenovirus overexpression of the wild-type caveolin-1 or delivery of caveolin-1 scaffolding domain peptide inside the cells decreased stretch-induced ERK phosphorylation and SP-C mRNA expression. In conclusion, our data suggest that caveolin-1 is present in E19 fetal type II epithelial cells. Caveolin-1 is translocated from the plasma membrane to the cytoplasm by mechanical stretch and functions as an inhibitory protein in stretch-induced type II cell differentiation via the ERK pathway.

3.1457 Variation of Physiochemical Properties and Cell Association Activity of Membrane Vesicles with Growth Phase in Pseudomonas aeruginosa

Tashiro, Y., Ichikawa, S., Shimizu, M., Toyofuka, M., Takaya, N., Nakajima-Kambe, T., Uchiyama, H. and Nomura, N.
Appl. Envr. Microbiol., ***76(11)***, *3732-3739 (2010)*

Pseudomonas aeruginosa and other Gram-negative bacteria release membrane vesicles (MVs) from their surfaces, and MVs have an ability to interact with bacterial cells. Although it has been known that many bacteria have mechanisms that control their phenotypes with the transition from exponential phase to stationary phase, changes of properties in released MVs have been poorly understood. Here, we demonstrate that MVs released by *P. aeruginosa* during the exponential and stationary phases possess different physiochemical properties. MVs purified from the stationary phase had higher buoyant densities than did those purified from the exponential phase. Surface charge, characterized by zeta potential, of MVs tended to be more negative as the growth shifted to the stationary phase, although the charges of PAO1 cells were not altered. *Pseudomonas* quinolone signal (PQS), one of the regulators related to MV production in *P. aeruginosa*, was lower in MVs purified from the exponential phase than in those from the stationary phase. MVs from the stationary phase more strongly associated with *P. aeruginosa* cells than did

those from the exponential phase. Our findings suggest that properties of MVs are altered to readily interact with bacterial cells along with the growth transition in *P. aeruginosa*. *T*

3.1458 **The Legs at odd angles (Loa) Mutation in Cytoplasmic Dynein Ameliorates Mitochondrial Function in SOD1G93A Mouse Model for Motor Neuron Disease**
El-Kadi, A.M., Bros-Facer, V., Deng, W., Philpott, A., Stoddart, E., Banks, G., Jackson, G.S., Fisher, E.M., Duchen, M.R., Greeensmith, L., Moore, A.L. and Hafezparast, M.
J. Biol. Chem., ***285(24)***, *18627-18639 (2010)*

Amyotrophic lateral sclerosis (ALS) is a debilitating and fatal late-onset neurodegenerative disease. Familial cases of ALS (FALS) constitute ~ 10% of all ALS cases, and mutant superoxide dismutase 1 (SOD1) is found in 15–20% of FALS. SOD1 mutations confer a toxic gain of unknown function to the protein that specifically targets the motor neurons in the cortex and the spinal cord. We have previously shown that the autosomal dominant *Legs at odd angles* (*Loa*) mutation in cytoplasmic dynein heavy chain (*Dync1h1*) delays disease onset and extends the life span of transgenic mice harboring human mutant $SOD1^{G93A}$. In this study we provide evidence that despite the lack of direct interactions between mutant SOD1 and either mutant or wild-type cytoplasmic dynein, the *Loa* mutation confers significant reductions in the amount of mutant SOD1 protein in the mitochondrial matrix. Moreover, we show that the *Loa* mutation ameliorates defects in mitochondrial respiration and membrane potential observed in $SOD1^{G93A}$ motor neuron mitochondria. These data suggest that the *Loa* mutation reduces the vulnerability of mitochondria to the toxic effects of mutant SOD1, leading to improved mitochondrial function in $SOD1^{G93A}$ motor neurons.

3.1459 **Modulation of intracellular trafficking by TGFB and rhoa**
Ribe, D. and Stenbeck, G.
Bone, ***47***, *Suppl. 1, S130-S131 (2010)*

Bone diseases such as osteoarthritis and osteoporosis are characterised by defects in transforming growth factor beta (TGFβ) signalling and matrix protein deposition. We are interested in the immediate early effects of TGFβ in matrix secretion. We are studying the trafficking and secretion of the matrix proteins osteonectin (ON) and osteopontin (OP) in osteoblasts using total internal reflection fluorescence microscopy (TIRFM) and membrane fractionation.
ROS 17/2.8 cells were transfected with a plasmid for expression of green fluorescent protein (GFP) – tagged ON and examined by TIRFM. Dynamics of ON-containing vesicles were studied by taking TIRFM images of individual cells at 0.3 s intervals for 30 s. Vesicles were identified and their trajectories tracked in ImageJ using the Particle Tracker plugin. Membrane fractions of non-transfected ROS 17/2.8 cells were separated by ultracentrifugation on an Optiprep density gradient and examined by Western blotting.
In unstimulated cells, a minority of ON-containing vesicles displayed sustained fast movement along their trajectories (150 nm in 3 s). Treatment of cells with 25 nM TGFβ increased the number of these faster vesicles by 34% within 10 min, an effect that was sustained 20 min after addition of TGFβ. Vesicle motility reverted to the unstimulated state within 60 min of TGFβ treatment.
Pre-treatment of cells for 60 min with 2.5 μM of the ROCK inhibitor Y-27632 reduced the mean vesicle speed and displacement by 28% and 43% respectively. Vesicle speed, displacement and the number of fast vesicles did not recover with addition of 25 nM TGFβ to Y-27632 –treated cells. Treatment with 10 μM nocodazole for 20 min completely inhibited vesicle motility, indicating that the microtubule network is essential for fidelity of membrane trafficking.
Western blotting of membrane fractions separated on a density gradient showed that blocking the endogenous TGFβ signal with the monoclonal TGFβ antibody mAb240 (1 μg/ml) for 60 min caused a shift in the distribution of OP from a vesicular localisation towards fractions rich in Golgi markers.
These studies show that TGFβ has immediate effects on trafficking of the secreted proteins ON and OP in osteoblasts, which are modulated by the Rho signalling cascade. These immediate early events in TGFβ signalling may play a role not only in matrix protein secretion but also in the propagation of further signals that lead to proliferation and aberrant matrix deposition if uncontrolled.

3.1460 **Towards a membrane proteome in *Drosophila*: a method for the isolation of plasma membrane**
Khanna, M.R., Stanley, B.A. and Thomas, G.H.
BMC Genomics, ***11***, *302-317 (2010)*

Background
The plasma membrane (PM) is a compartment of significant interest because cell surface proteins influence the way in which a cell interacts with its neighbours and its extracellular environment. However, PM is hard to isolate because of its low abundance. Aqueous two-phase affinity purification (2PAP), based on PEG/Dextran two-phase fractionation and lectin affinity for PM-derived microsomes, is an emerging method for the isolation of high purity plasma membranes from several vertebrate sources. In contrast, PM isolation techniques in important invertebrate genetic model systems, such as *Drosophila melanogaster*, have relied upon enrichment by density gradient centrifugation. To facilitate genetic investigation of activities contributing to the content of the PM sub-proteome, we sought to adapt 2PAP to this invertebrate model to provide a robust PM isolation technique for *Drosophila*.
Results
We show that 2PAP alone does not completely remove contaminating endoplasmic reticulum and mitochondrial membrane. However, a novel combination of density gradient centrifugation plus 2PAP results in a robust PM preparation. To demonstrate the utility of this technique we isolated PM from fly heads and successfully identified 432 proteins using MudPIT, of which 37% are integral membrane proteins from all compartments. Of the 432 proteins, 22% have been previously assigned to the PM compartment, and a further 34% are currently unassigned to any compartment and represent candidates for assignment to the PM. The remainder have previous assignments to other compartments.
Conclusion
A combination of density gradient centrifugation and 2PAP results in a robust, high purity PM preparation from *Drosophila*, something neither technique can achieve on its own. This novel preparation should lay the groundwork for the proteomic investigation of the PM in different genetic backgrounds in *Drosophila*. Our results also identify two key steps in this procedure: The optimization of membrane partitioning in the PEG/Dextran mixture, and careful choice of the correct lectin for the affinity purification step in light of variations in bulk membrane lipid composition and glycosylation patterns respectively. This points the way for further adaptations into other systems.

3.1461 Epstein-Barr Virus Latent Membrane Protein 1 Modulates Distinctive NF-κB Pathways through C-Terminus-Activating Region 1 To Regulate Epidermal Growth Factor Receptor Expression
Kung, C-P. and Raab-Traub, N.
J. Virol., ***84(13)***, *6605-6614 (2010)*

Epstein-Barr Virus (EBV) latent membrane protein 1 (LMP1) is required for EBV B-lymphocyte transformation, transforms rodent fibroblasts, and can induce lymphoma and epithelial hyperplasia in transgenic mice. Two domains have been identified within the intracellular carboxy terminus that can activate NF-κB, C-terminus-activating region 1 (CTAR1) and CTAR2, through interactions with tumor necrosis receptor-associated factors (TRAFs). CTAR1 can activate both the canonical and noncanonical NF-κB pathways and has unique effects on cellular gene expression. The epidermal growth factor receptor (EGFR) is highly induced by LMP1-CTAR1 in epithelial cells through activation of a novel NF-κB form containing p50 homodimers and Bcl-3. To further understand the regulation of NF-κB in CTAR1-induced EGFR expression, we evaluated the ability of CTAR1 to induce EGFR in mouse embryonic fibroblasts (MEFs) defective for different NF-κB effectors. CTAR1-mediated EGFR induction required the NF-κB-inducing kinase (NIK) but not the IκB kinase (IKK) complex components that regulate canonical or noncanonical NF-κB pathways. CTAR1-mediated induction of nuclear p50 occurred in IKKβ-, IKKγ-, and NIK-defective MEFs, indicating that this induction is not dependent on the canonical or noncanonical NF-κB pathways. EGFR and nuclear p50 were expressed at high levels in TRAF2–/– fibroblasts and were not induced by CTAR1. In TRAF3–/– MEFs, CTAR1 induced nuclear p50 but did not affect basal levels of STAT3 serine phosphorylation or induce EGFR expression. EGFR was induced by LMP1 in TRAF6–/– MEFs. These findings suggest that this novel NF-κB pathway is differentially regulated by TRAF2 and TRAF3, and that distinct interactions of LMP1 and its effectors regulate LMP1-mediated gene expression.

3.1462 Demonstration of a Direct Interaction between σ-1 Receptors and Acid-Sensing Ion Channels
Carnally, S.M., Johannessen, M., Henderson, R.M., Jackson, M.B. and Edwardsen, J.M.
Biophys. J., ***98***, *1182-1191 (2010)*

The σ-1 receptor is a widely expressed protein that interacts with a variety of ion channels, including the acid-sensing ion channel (ASIC) 1a. Here we used atomic force microscopy to determine the architecture of the ASIC1a/σ-1 receptor complex. When isolated His_8-tagged ASIC1a was imaged in complex with anti-His_6 antibodies, the angle between pairs of bound antibodies was 135°, consistent with the known trimeric structure of the channel. When ASIC1a was coexpressed with FLAG/His_6-tagged σ-1 receptor,

ASIC1a became decorated with small particles, and pairs of these particles bound at an angle of 131°. When these complexes were incubated with anti-FLAG antibodies, pairs of antibodies bound at an angle of 134°, confirming that the small particles were σ-1 receptors. Of interest, we found that the σ-1 receptor ligand haloperidol caused an ~50% reduction in ASIC1a/σ-receptor binding, suggesting a way in which σ-1 ligands might modulate channel properties. For the first time, to our knowledge, we have resolved the structure of a complex between the σ-1 receptor and a target ion channel, and demonstrated that the stoichiometry of the interaction is 1 σ-1 receptor/1 ASIC1a subunit.

3.1463 Analysis of SM4 sulfatide as a P-selectin ligand using model membranes
Simonis, D., Schlesinger, M.-, Seelandt, C., Borsig, L. and Bendas, G.
Biophys. Chem., ***150***, *98-104 (2010)*

Carcinoma tumor cells express highly glycosylated mucins acting as ligands for selectin adhesion receptors and thus facilitating the metastatic process. Recently, a sulfated galactocerebroside SM4 was detected as solely P-selectin ligand on MC-38 colon carcinoma cells. Here we characterize the functionality of SM4 as selectin ligand using model membrane approaches. SM4 was found concentrated in lipid rafts of MC-38 cells indicating a local clustering that may increase the avidity of P-selectin recognition. To confirm this, SM4 was incorporated at various concentrations into POPC model membranes and lateral clustering was analyzed by fluorescence microscopy and found to be comparable to glycolipids carrying the sLe^x epitope. SM4 containing liposomes were used as cell models, binding to immobilized P-selectin. Quartz crystal microbalance data confirmed SM4/P-selectin liposome binding that was inhibited dose-dependently by heparin. Comparable binding characteristics of SM4 and sLe^x liposomes underscore the similarity of these epitopes. Thus, clustering of SM4 on tumor cells is a principle for binding P-selectin.

3.1464 Live cell visualization of the interactions between HIV-1 Gag and the cellular RNA-binding protein Staufen1
Milev, M.P., Brown, C.M. and Mouland, A.J.
Retrovirology, ***7***, *41-59 (2010)*

Background
Human immunodeficiency virus type 1 (HIV-1) uses cellular proteins and machinery to ensure transmission to uninfected cells. Although the host proteins involved in the transport of viral components toward the plasma membrane have been investigated, the dynamics of this process remain incompletely described. Previously we showed that the double-stranded (ds)RNA-binding protein, Staufen1 is found in the HIV-1 ribonucleoprotein (RNP) that contains the HIV-1 genomic RNA (vRNA), Gag and other host RNA-binding proteins in HIV-1-producing cells. Staufen1 interacts with the nucleocapsid domain (NC) domain of Gag and regulates Gag multimerization on membranes thereby modulating HIV-1 assembly. The formation of the HIV-1 RNP is dynamic and likely central to the fate of the vRNA during the late phase of the HIV-1 replication cycle.
Results
Detailed molecular imaging of both the intracellular trafficking of virus components and of virus-host protein complexes is critical to enhance our understanding of factors that contribute to HIV-1 pathogenesis. In this work, we visualized the interactions between Gag and host proteins using bimolecular and trimolecular fluorescence complementation (BiFC and TriFC) analyses. These methods allow for the direct visualization of the localization of protein-protein and protein-protein-RNA interactions in live cells. We identified where the virus-host interactions between Gag and Staufen1 and Gag and IMP1 (also known as VICKZ1, IGF2BP1 and ZBP1) occur in cells. These virus-host interactions were not only detected in the cytoplasm, but were also found at cholesterol-enriched GM1-containing lipid raft plasma membrane domains. Importantly, Gag specifically recruited Staufen1 to the detergent insoluble membranes supporting a key function for this host factor during virus assembly. Notably, the TriFC experiments showed that Gag and Staufen1 actively recruited protein partners when tethered to mRNA.
Conclusions
The present work characterizes the interaction sites of key components of the HIV-1 RNP (Gag, Staufen1 and IMP1), thereby bringing to light where HIV-1 recruits and co-opts RNA-binding proteins during virus assembly.

3.1465 Termination of autophagy and reformation of lysosomes regulated by mTOR
Yu, L., McPhee, C.K., Zheng, L., Mardones, G.A., Rong, Y., Peng, J., Mi, N., Zhao, Y., Liu, Z., Wan, F., Hailey, D.W., Oorscot, V., Klumperman, J., Baehrecke, E.H. and Lenardo, M.J.
Nature, ***465(17)***, *942-946 (2010)*

Autophagy is an evolutionarily conserved process by which cytoplasmic proteins and organelles are catabolized[1, 2]. During starvation, the protein TOR (target of rapamycin), a nutrient-responsive kinase, is inhibited, and this induces autophagy. In autophagy, double-membrane autophagosomes envelop and sequester intracellular components and then fuse with lysosomes to form autolysosomes, which degrade their contents to regenerate nutrients. Current models of autophagy terminate with the degradation of the autophagosome cargo in autolysosomes[3, 4, 5], but the regulation of autophagy in response to nutrients and the subsequent fate of the autolysosome are poorly understood. Here we show that mTOR signalling in rat kidney cells is inhibited during initiation of autophagy, but reactivated by prolonged starvation. Reactivation of mTOR is autophagy-dependent and requires the degradation of autolysosomal products. Increased mTOR activity attenuates autophagy and generates proto-lysosomal tubules and vesicles that extrude from autolysosomes and ultimately mature into functional lysosomes, thereby restoring the full complement of lysosomes in the cell—a process we identify in multiple animal species. Thus, an evolutionarily conserved cycle in autophagy governs nutrient sensing and lysosome homeostasis during starvation.

3.1466 Coronaviruses Hijack the LC3-I-Positive EDEMosomes, ER-Derived Vesicles Exporting Short-Lived ERAD Regulators, for Replication

Reggiori, F., Monastyrska, I., Verheiji, M.H., Cali, T., Ulasli, M., Bianchi, S., Bernasconi, R., de Haan, C.A.M. and Molinari, M.
*Cell Host & Microbe, **7**, 500-508 (2010)*

Coronaviruses (CoV), including SARS and mouse hepatitis virus (MHV), are enveloped RNA viruses that induce formation of double-membrane vesicles (DMVs) and target their replication and transcription complexes (RTCs) on the DMV-limiting membranes. The DMV biogenesis has been connected with the early secretory pathway. CoV-induced DMVs, however, lack conventional endoplasmic reticulum (ER) or Golgi protein markers, leaving their membrane origins in question. We show that MHV co-opts the host cell machinery for COPII-independent vesicular ER export of a short-living regulator of ER-associated degradation (ERAD), EDEM1, to derive cellular membranes for replication. MHV infection causes accumulation of EDEM1 and OS-9, another short-living ER chaperone, in the DMVs. DMVs are coated with the nonlipidated LC3/Atg8 autophagy marker. Downregulation of LC3, but not inactivation of host cell autophagy, protects cells from CoV infection. Our study identifies the host cellular pathway hijacked for supplying CoV replication membranes and describes an autophagy-independent role for nonlipidated LC3-I.

3.1467 Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes

Oberle, C., Huai, J., Reinheckel, T., Tacke, M., Rassner, M., Ekert, P.G., Buellesbach, J. and Borner, C.
*Cell Death and Differentiation, **17**, 1167-1178 (2010)*

Apoptotic stimuli have been shown to trigger lysosomal membrane permeability (LMP), leading to the release of cathepsins, which activate death signaling pathways in the cytosol. However, it is unknown whether this process is an initiating or amplifying event in apoptosis. In this study, we used fibroblasts and monocytes exposed to etoposide, ultraviolet light, FasL or deprived of interleukin-3 (IL-3) to show that LMP and the cytosolic release of cathepsins B, L and D consistently depends on Bax/Bak and components of the apoptosome. Neither Bax nor Bak resided on the lysosomes, indicating that lysosomes were not directly perforated by Bax/Bak but by effectors downstream of the apoptosome. Detailed kinetic analysis of cells lacking cathepsin B or L or treated with the cysteine protease inhibitor, E64d, revealed a delay in these cells in etoposide- and IL-3 deprivation-induced caspase-3 activation and apoptosis induction but not clonogenic survival, indicating that cathepsins amplify rather than initiate apoptosis.

3.1468 Non-classical exocytosis of [alpha]-synuclein is sensitive to folding states and promoted under stress conditions

Jang, A., Lee, H-L., Suk, J-E., Jung, J-W., Kim, K-P. and Lee, S-J.
*J. Neurochem., **113(5)**, 1263-1274 (2010)*

Parkinson's disease is characterized by deposition of misfolded/aggregated [alpha]-synuclein proteins in multiple regions of the brain. Neurons can release [alpha]-synuclein; through this release, pathological forms of [alpha]-synuclein are propagated between neurons, and also cause neuroinflammation. In this study, we demonstrate that release of [alpha]-synuclein is consistently increased under various protein

misfolding stress conditions in both neuroblastoma and primary neuron models. This release is mediated by a non-classical, endoplasmic reticulum (ER)/Golgi-independent exocytosis, and stress-induced release coincides with increased translocation of [alpha]-synuclein into vesicles. Both vesicle translocation and secretion were blocked by attachment of a highly stable, globular protein to [alpha]-synuclein, whereas forced protein misfolding resulted in an increase in both of these activities. Mass spectrometry analysis showed a higher degree of oxidative modification in secreted [alpha]-synuclein than in the cellular protein. Together, these results suggest that structurally abnormal, damaged [alpha]-synuclein proteins translocate preferentially into vesicles and are released from neuronal cells via exocytosis.

3.1469 The fibroblast growth factor receptor substrate 3 adapter is a developmentally regulated microtubule-associated protein expressed in migrating and differentiated neurons
Hryciw, T., MacDonald, J.I.S., Phillips, R., Seah, C., Pasternak, S. and Meakin, S.O.
J. Neurochem., ***112***, *924-939 (2010)*

Fibroblast growth factor (FGF) mediated signaling is essential to many aspects of neural development. Activated FGF receptors signal primarily through the FGF receptor substrate (Frs) adapters, which include Frs2/Frs2[alpha] and Frs3/Frs2[beta]. While some studies suggest that Frs3 can compensate for the loss of Frs2 in transfected cells, the lack of an effective Frs3 specific antibody has prevented efforts to determine the role(s) of the endogenous protein. To this end, we have generated a Frs3 specific antibody and have characterized the pattern of Frs3 expression in the developing nervous system, its subcellular localization as well as its biochemical properties. We demonstrate that Frs3 is expressed at low levels in the ventricular zone of developing cortex, between E12 and E15, and it co-localizes with nestin and acetylated [alpha]-tubulin in radial processes in the ventricular/subventricular zones as well as with [beta]III tubulin in differentiated cortical neurons. Subcellular fractionation studies demonstrate that endogenous Frs3 is both soluble and plasma membrane associated while Frs3 expressed in 293T cells associates exclusively with lipid rafts. Lastly, we demonstrate that neuronal Frs3 binds microtubules comparable to the microtubule-associated protein, MAP2, while Frs2 does not. Collectively, these data suggest that neuronal Frs3 functions as a novel microtubule binding protein and they provide the first biochemical evidence that neuronal Frs3 is functionally distinct from Frs2/Frs2[alpha].

3.1470 Characterization of a novel organelle in Toxoplasma gondii with similar composition and function to the plant vacuole
Miranda, K., Pace, D.A., Cintron, R., Rodrigues, J.C.F., fang, J., Smith, A., Rohloff, P., Coelho, E., de Haas, F., de Souza, W., Coppens, I., Sibley, L.D. and Moreno, S.N.J.
Mol. Microbiol., ***76(6)***, *1358-1375 (2010)*

Toxoplasma gondii belongs to the phylum Apicomplexa and is an important cause of congenital disease and infection in immunocompromised patients. Like most apicomplexans, T. gondii possesses several plant-like features, such as the chloroplast-like organelle, the apicoplast. We describe and characterize a novel organelle in T. gondii tachyzoites, which is visible by light microscopy and possesses a broad similarity to the plant vacuole. Electron tomography shows the interaction of this vacuole with other organelles. The presence of a plant-like vacuolar proton pyrophosphatase (TgVP1), a vacuolar proton ATPase, a cathepsin L-like protease (TgCPL), an aquaporin (TgAQP1), as well as Ca2+/H+ and Na+/H+ exchange activities, supports similarity to the plant vacuole. Biochemical characterization of TgVP1 in enriched fractions shows a functional similarity to the respective plant enzyme. The organelle is a Ca2+ store and appears to have protective effects against salt stress potentially linked to its sodium transport activity. In intracellular parasites, the organelle fragments, with some markers colocalizing with the late endosomal marker, Rab7, suggesting its involvement with the endocytic pathway. Studies on the characterization of this novel organelle will be relevant to the identification of novel targets for chemotherapy against T. gondii and other apicomplexan parasites as well.

3.1471 Rab11 Supports Amphetamine-Stimulated Norepinephrine Transporter Trafficking
Matthies, H.J.G., Moore, J.L., Saunders, C., Matthies, D.S., Lapierre, L.A., Goldenring, J.R., Blakely, R.D. and Galli, A.
J. Neurosci., ***30(23)***, *7863-7877 (2010)*

The norepinephrine transporter (NET) is a presynaptic plasma membrane protein that mediates reuptake of synaptically released norepinephrine. NET is also a major target for medications used for the treatment of depression, attention deficit/hyperactivity disorder, narcolepsy, and obesity. NET is regulated by numerous mechanisms, including catalytic activation and membrane trafficking. Amphetamine (AMPH), a

psychostimulant and NET substrate, has also been shown to induce NET trafficking. However, neither the molecular basis nor the nature of the relevant membrane compartments of AMPH-modulated NET trafficking has been defined. Indeed, direct visualization of drug-modulated NET trafficking in neurons has yet to be demonstrated. In this study, we used a recently developed NET antibody and the presence of large presynaptic boutons in sympathetic neurons to examine basal and AMPH-modulated NET trafficking. Specifically, we establish a role for Rab11 in AMPH-induced NET trafficking. First, we found that, in cortical slices, AMPH induces a reduction in surface NET. Next, we observed AMPH-induced accumulation and colocalization of NET with Rab11a and Rab4 in presynaptic boutons of cultured neurons. Using tagged proteins, we demonstrated that NET and a truncated Rab11 effector (FIP2ΔC2) do not redistribute in synchrony, whereas NET and wild-type Rab11a do. Analysis of various Rab11a/b mutants further demonstrates that Rab11 regulates NET trafficking. Expression of the truncated Rab11a effector (FIP2ΔC2) attenuates endogenous Rab11 function and prevented AMPH-induced NET internalization as does GDP-locked Rab4 S22N. Our data demonstrate that AMPH leads to an increase of NET in endosomes of single boutons and varicosities in a Rab11-dependent manner.

3.1472 Nox4-Derived H2O2 Mediates Endoplasmic Reticulum Signaling through Local Ras Activation
Wu, R-F., Ma, Z., Liu, Z. and Terada, L.S.
Mol. Cell. Biol., ***30(14)***, *3553-3568 (2010)*

The unfolded-protein response (UPR) of the endoplasmic reticulum (ER) has been linked to oxidant production, although the molecular details and functional significance of this linkage are poorly understood. Using a ratiometric H_2O_2 sensor targeted to different subcellular compartments, we demonstrate specific production of H_2O_2 by the ER in response to the stressors tunicamycin and HIV-1 Tat, but not to thapsigargin or dithiothreitol. Knockdown of the oxidase Nox4, expressed on ER endomembranes, or expression of ER-targeted catalase blocked ER H_2O_2 production by tunicamycin and Tat and prevented the UPR following exposure to these two agonists, but not to thapsigargin or dithiothreitol. Tat also triggered Nox4-dependent, sustained activation of Ras leading to ERK, but not phosphatidylinositol 3-kinase (PI3K)/mTOR, pathway activation. Cell fractionation studies and green fluorescent protein (GFP) fusions of GTPase effector binding domains confirmed selective activation of endogenous RhoA and Ras on the ER surface, with ER-associated K-Ras acting upstream of the UPR and downstream of Nox4. Notably, the Nox4/Ras/ERK pathway induced autophagy, and suppression of autophagy unmasked cell death and prevented differentiation of endothelial cells in 3-dimensional matrix. We conclude that the ER surface provides a platform to spatially organize agonist-specific Nox4-dependent oxidative signaling events, leading to homeostatic protective mechanisms rather than oxidative stress.

3.1473 Lysosomal Proteolysis and Autophagy Require Presenilin 1 and Are Disrupted by Alzheimer-Related PS1 Mutations
Lee, J-H., Yu, W.H., Kumar, A., Lee, S., Mohan, P.S., Peterhoff, C.M., Wolfe, D.M., Martinez-Vicente, M., Massey, A.C., Sovak, G., Uchiyama, Y., Westaway, D., Cuervo, A.M. and Nixon, R.A.
Cell, ***141***, *1146-1158 (2010)*

Macroautophagy is a lysosomal degradative pathway essential for neuron survival. Here, we show that macroautophagy requires the Alzheimer's disease (AD)-related protein presenilin-1 (PS1). In PS1 null blastocysts, neurons from mice hypomorphic for PS1 or conditionally depleted of PS1, substrate proteolysis and autophagosome clearance during macroautophagy are prevented as a result of a selective impairment of autolysosome acidification and cathepsin activation. These deficits are caused by failed PS1-dependent targeting of the v-ATPase V0a1 subunit to lysosomes. N-glycosylation of the V0a1 subunit, essential for its efficient ER-to-lysosome delivery, requires the selective binding of PS1 holoprotein to the unglycosylated subunit and the Sec61alpha/oligosaccharyltransferase complex. PS1 mutations causing early-onset AD produce a similar lysosomal/autophagy phenotype in fibroblasts from AD patients. PS1 is therefore essential for v-ATPase targeting to lysosomes, lysosome acidification, and proteolysis during autophagy. Defective lysosomal proteolysis represents a basis for pathogenic protein accumulations and neuronal cell death in AD and suggests previously unidentified therapeutic targets.

3.1474 Activation of Adenosine A2A Receptors Induces TrkB Translocation and Increases BDNF-Mediated Phospho-TrkB Localization in Lipid Rafts: Implications for Neuromodulation
Assaife-Lopes, N., Sousa, V.C., Pereira, D.B., Ribeiro, J.A., Chao, M.V. and Sebastiao, A.M.
J. Neurosci., ***30(25)***, *8468-8480 (2010)*

Brain-derived neurotrophic factor (BDNF) signaling is critical for neuronal development and transmission. Recruitment of TrkB receptors to lipid rafts has been shown to be necessary for the activation of specific signaling pathways and modulation of neurotransmitter release by BDNF. Since TrkB receptors are known to be modulated by adenosine A_{2A} receptor activation, we hypothesized that activation of A_{2A} receptors could influence TrkB receptor localization among different membrane microdomains. We found that adenosine A_{2A} receptor agonists increased the levels of TrkB receptors in the lipid raft fraction of cortical membranes and potentiated BDNF-induced augmentation of phosphorylated TrkB levels in lipid rafts. Blockade of the clathrin-mediated endocytosis with monodansylcadaverine (100 μM) did not modify the effects of the A_{2A} receptor agonists but significantly impaired BDNF effects on TrkB recruitment to lipid rafts. The effect of A_{2A} receptor activation in TrkB localization was mimicked by 5 μM forskolin, an adenylyl cyclase activator. Also, it was blocked by the PKA inhibitors Rp-cAMPs and PKI-(14–22), and by the Src-family kinase inhibitor PP2. Moreover, removal of endogenous adenosine or disruption of lipid rafts reduced BDNF stimulatory effects on glutamate release from cortical synaptosomes. Lipid raft integrity was also required for the effects of BDNF on hippocampal long-term potentiation at CA1 synapses. Our data demonstrate, for the first time, a BDNF-independent recruitment of TrkB receptors to lipid rafts induced by activation of adenosine A_{2A} receptors, with functional consequences for TrkB phosphorylation and BDNF-induced modulation of neurotransmitter release and hippocampal plasticity.

3.1475 The Platelet Actin Cytoskeleton Associates with SNAREs and Participates in α-Granule Secretion
Woronowicz, K., Dilks, J.R., Rozenvayn, N., Dowal, L., Blair, P.S., Peters, C.G., Woronowicz, L. and Flaumenhaft, R.
Biochemistry, ***49(21)***, *4533-4542 (2010)*

Following platelet activation, platelets undergo a dramatic shape change mediated by the actin cytoskeleton and accompanied by secretion of granule contents. While the actin cytoskeleton is thought to influence platelet granule secretion, the mechanism for this putative regulation is not known. We found that disruption of the actin cytoskeleton by latrunculin A inhibited α-granule secretion induced by several different platelet agonists without significantly affecting activation-induced platelet aggregation. In a cell-free secretory system, platelet cytosol was required for α-granule secretion. Inhibition of actin polymerization prevented α-granule secretion in this system, and purified platelet actin could substitute for platelet cytosol to support α-granule secretion. To determine whether SNAREs physically associate with the actin cytoskeleton, we isolated the Triton X-100 insoluble actin cytoskeleton from platelets. VAMP-8 and syntaxin-2 associated only with actin cytoskeletons of activated platelets. Syntaxin-4 and SNAP-23 associated with cytoskeletons isolated from either resting or activated platelets. When syntaxin-4 and SNAP-23 were tested for actin binding in a purified protein system, only syntaxin-4 associated directly with polymerized platelet actin. These data show that the platelet cytoskeleton interacts with select SNAREs and that actin polymerization facilitates α-granule release.

3.1476 Superoxide dismutase-1 and other proteins in inclusions from transgenic amyotrophic lateral sclerosis model mice
Bergemalm, D., Forsberg, K., Srivastava, V., Graffmo, K.S., Andersen, P.M., Brännström, T., Wingsle, G. and Marklund, S.L.
J. Neurochem.,***114***, *408-418 (2010)*

Mutant superoxide dismutase-1 (SOD1) causes amyotrophic lateral sclerosis (ALS) through a cytotoxic mechanism of unknown nature. A hallmark in ALS patients and transgenic mouse models carrying human SOD1 (hSOD1) mutations are hSOD1-immunoreactive inclusions in spinal cord ventral horns. The hSOD1 inclusions may block essential cellular functions or cause toxicity through sequestering of other proteins. Inclusions from four different transgenic mouse models were examined after density gradient ultracentrifugation. The inclusions are complex structures with heterogeneous densities and are disrupted by detergents. The aggregated hSOD1 was mainly composed of subunits that lacked the native stabilizing intra-subunit disulfide bond. A proportion of subunits formed hSOD1 oligomers or was bound to other proteins through disulfide bonds. Dense inclusions could be isolated and the protein composition was analyzed using proteomic techniques. Mutant hSOD1 accounted for half of the protein. Ten other proteins were identified. Two were cytoplasmic chaperones, four were cytoskeletal proteins, and 4 were proteins that normally reside in the endoplasmic reticulum (ER). The presence of ER proteins in inclusions containing the primarily cytosolic hSOD1 further supports the notion that ER stress is involved in ALS.

3.1477 Unconventional Secretion of AcbA in Dictyostelium discoideum through a Vesicular Intermediate
Cabral, M., Anjard, C., Malhotra, V., Loomis, W.F. and Kuspa, A.

*Eukaryot. Cell, **9(7)**, 1009-1017 (2010)*

The acyl coenzyme A (CoA) binding protein AcbA is secreted unconventionally and processed into spore differentiation factor 2 (SDF-2), a peptide that coordinates sporulation in Dictyostelium discoideum. We report that AcbA is localized in vesicles that accumulate in the cortex of prespore cells just prior to sporulation. These vesicles are not observed after cells are stimulated to release AcbA but remain visible after stimulation in cells lacking the Golgi reassembly stacking protein (GRASP). Acyl-CoA binding is required for the inclusion of AcbA in these vesicles, and the secretion of AcbA requires N-ethylmaleimide-sensitive factor (NSF). About 1% of the total cellular AcbA can be purified within membrane-bound vesicles. The yield of vesicles decreases dramatically when purified from wild-type cells that were stimulated to release AcbA, whereas the yield from GRASP mutant cells was only modestly altered by stimulation. We suggest that these AcbA-containing vesicles are secretion intermediates and that GRASP functions at a late step leading to the docking/fusion of these vesicles at the cell surface.

3.1478 Reduction of Brain β-Amyloid (Aβ) by Fluvastatin, a Hydroxymethylglutaryl-CoA Reductase Inhibitor, through Increase in Degradation of Amyloid Precursor Protein C-terminal Fragments (APP-CTFs) and Aβ Clearance

Shinohaa, M., Sato, N., Kurinami, H., Takeuchi, D., Takeda, S., Shimamura, M., Yamashita, T., Uchiyama, Y., Rakugi, H. and Morishita, R.
*J. Biol. Chem., **285(29)**, 22091-22102 (2010)*

Epidemiological studies suggest that statins (hydroxymethylglutaryl-CoA reductase inhibitors) could reduce the risk of Alzheimer disease. Although one possible explanation is through an effect on β-amyloid (Aβ) metabolism, its effect remains to be elucidated. Here, we explored the molecular mechanisms of how statins influence Aβ metabolism. Fluvastatin at clinical doses significantly reduced Aβ and amyloid precursor protein C-terminal fragment (APP-CTF) levels among APP metabolites in the brain of C57BL/6 mice. Chronic intracerebroventricular infusion of lysosomal inhibitors blocked these effects, indicating that up-regulation of the lysosomal degradation of endogenous APP-CTFs is involved in reduced Aβ production. Biochemical analysis suggested that this was mediated by enhanced trafficking of APP-CTFs from endosomes to lysosomes, associated with marked changes of Rab proteins, which regulate endosomal function. In primary neurons, fluvastatin enhanced the degradation of APP-CTFs through an isoprenoid-dependent mechanism. Because our previous study suggests additive effects of fluvastatin on Aβ metabolism, we examined Aβ clearance rates by using the brain efflux index method and found its increased rates at high Aβ levels from brain. As LRP1 in brain microvessels was increased, up-regulation of LRP1-mediated Aβ clearance at the blood-brain barrier might be involved. In cultured brain microvessel endothelial cells, fluvastatin increased LRP1 and the uptake of Aβ, which was blocked by LRP1 antagonists, through an isoprenoid-dependent mechanism. Overall, the present study demonstrated that fluvastatin reduced Aβ level by an isoprenoid-dependent mechanism. These results have important implications for the development of disease-modifying therapy for Alzheimer disease as well as understanding of Aβ metabolism.

3.1479 Modulation of the Protein Kinase Cδ Interaction with the "d" Subunit of F1F0-ATP Synthase in Neonatal Cardiac Myocytes: DEVELOPMENT OF CELL-PERMEABLE, MITOCHONDRIALLY TARGETED INHIBITOR AND FACILITATOR PEPTIDES

Nguyen,, T.T., Ogbi, M., Yu, Q., Fishman, J.B., Thomas, W., Harvey, B.J., Fulton, D. and Johnson, J.A.
*J. Biol. Chem., **285(29)**, 22164-22173 (2010)*

The F_1F_0-ATP synthase provides ~ 90% of cardiac ATP, yet little is known regarding its regulation under normal or pathological conditions. Previously, we demonstrated that protein kinase Cδ (PKCδ) inhibits F_1F_0 activity via an interaction with the "d" subunit of F_1F_0-ATP synthase (dF_1F_0) in neonatal cardiac myocytes (NCMs) (Nguyen, T., Ogbi, M., and Johnson, J. A. (2008) *J. Biol. Chem.* 283, 29831–29840). We have now identified a dF_1F_0-derived peptide (NH_2-^{2}AGRKLALKTIDWVSF^{16}-COOH) that inhibits PKCδ binding to dF_1F_0 in overlay assays. We have also identified a second dF_1F_0-derived peptide (NH_2-^{111}RVREYEKQLEKIKNMI^{126}-COOH) that facilitates PKCδ binding to dF_1F_0. Incubation of NCMs with versions of these peptides containing HIV-Tat protein transduction and mammalian mitochondrial targeting sequences resulted in their delivery into mitochondria. Preincubation of NCMs, with 10 nM extracellular concentrations of the mitochondrially targeted PKCδ-dF_1F_0 interaction inhibitor, decreased 100 nM 4β-phorbol 12-myristate 13-acetate (4β-PMA)-induced co-immunoprecipitation of PKCδ with dF_1F_0 by 50 ± 15% and abolished the 30 nM 4β-PMA-induced inhibition of F_1F_0-ATPase activity. A scrambled sequence (inactive) peptide, which contained HIV-Tat and mitochondrial targeting sequences,

was without effect. In contrast, the cell-permeable, mitochondrially targeted PKCδ-dF_1F_0 facilitator peptide by itself induced the PKCδ-dF_1F_0 co-immunoprecipitation and inhibited F_1F_0-ATPase activity. In *in vitro* PKC add-back experiments, the PKCδ-F_1F_0 inhibitor blocked PKCδ-mediated inhibition of F_1F_0-ATPase activity, whereas the facilitator induced inhibition. We have developed the first cell-permeable, mitochondrially targeted modulators of the PKCδ-dF_1F_0 interaction in NCMs. These novel peptides will improve our understanding of cardiac F_1F_0 regulation and may have potential as therapeutics to attenuate cardiac injury.

3.1480 A Toxin-based Probe Reveals Cytoplasmic Exposure of Golgi Sphingomyelin
Bakrac, B., Kladnik, A., Macek, P., McHaffie, G., Werner, A., Lakey, J.H. and Anderluh, G.
J. Biol. Chem., ***285(29)***, *22186-22195 (2010)*

Although sphingomyelin is an important cellular lipid, its subcellular distribution is not precisely known. Here we use a sea anemone cytolysin, equinatoxin II (EqtII), which specifically binds sphingomyelin, as a new marker to detect cellular sphingomyelin. A purified fusion protein composed of EqtII and green fluorescent protein (EqtII-GFP) binds to the SM rich apical membrane of Madin-Darby canine kidney (MDCK) II cells when added exogenously, but not to the SM-free basolateral membrane. When expressed intracellularly within MDCK II cells, EqtII-GFP colocalizes with markers for Golgi apparatus and not with those for nucleus, mitochondria, endoplasmic reticulum or plasma membrane. Colocalization with the Golgi apparatus was confirmed by also using NIH 3T3 fibroblasts. Moreover, EqtII-GFP was enriched in *cis*-Golgi compartments isolated by gradient ultracentrifugation. The data reveal that EqtII-GFP is a sensitive probe for membrane sphingomyelin, which provides new information on cytosolic exposure, essential to understand its diverse physiological roles.

3.1481 Targeting intracellular Aβ Oligomers through conformational intrabodies
Lecci, A. and Cattaneo, A.,
Alzheimer's and Dementia, ***6(4)****, Suppl. 1, S252-S253 (2010)*

Background: Increasing evidence supports the role of AβOligomers (AβOs) in the pathogenesis of Alzheimer's Disease (AD). The use of specific conformational intracellular antibodies (intrabodies) allows to selectively target AβOs inside cells at different points for mechanistic as well as for interference purposes. We previously described the selection of conformation-sensitive and sequence-specific single chain Fv antibody fragments (scFvs), targeting AβOs (Meli et al., 2009). Now, we describe the use of these scFvs as intrabodies for selective subcellular targeting and intracellular knock-down/silencing of AβOs, in well established cellular models for AβOs production and secretion. **Methods:** Sensitive co-immunoprecipitation methods allowed verifying the binding of the recombinant scFvs to the natural AβOs derived from cell culture media or from human Cerospinal Fluid (CSF). The intracellular expression of the anti-AβOs intrabodies was performed in a well established cell model for AβOs production and secretion, referred to as 7PA2 cells (Walsh et al.,2000;2002), generating cells stably transfected with intrabodies in different formats.The *in vivo* binding and the modulation of AβOs generation were studied by experiments of subcellular fractionation on discontinuous iodixanol gradients and by intrabody-AβOs co-immunoprecipitation approaches. **Results:** The recombinant anti-AβOs scFvs selectively bind and immunoprecipitate the natural AβOs from 7PA2 conditioned media and from human Cerospinal Fluid (CSF). The effects of the intracellular expression of the anti-AβOs intrabodies on the Aβ/AβOs formation and APP metabolism were studied by subcellular fractionation. Total microsomes fractionated from 7PA2 or 7PA2 expressing intrabodies showed differential distribution of Aβ/AβOs. Moreover, a sensitive intrabody-AβOs co-immunoprecipitation approach allowed demonstrating a selective immunoprecipitation of specific forms of natural SDS-stable AβOs.The expression of an intrabody with an ER-retention signal (KDEL) showed a significant reduction of AβOs secretion and selective modulation of AβOs accumulation in subcellular fractions. **Conclusions:** The binding ability of the anti-AβOs scFvs to the natural AβOs *in vitro* as well as *in vivo* (in 7PA2 living cells), validates their conformation-specificity and -selectivity. Moreover, the anti-AβOs intrabodies interfere with AβOs intracellular formation and secretion. The intrabody approach is therefore a promising way for interfering with the activity of different intracellular Aβ assemblies (in ways that would not be possible by RNA-interference approaches) exploitable for new recombinant therapeutics.

3.1482 ELGA stimulates gamma-secretase activity to increase Aβ generation
Jung, S.
Alzheimer's and dementia, ***6(4)****, Suppl. 1, S260 (2010)*

Background: Amyloid beta is the main pathologic hallmark of Alzheimer's Disease. This Hydrophobic fragment forms amyloid plaque and causes neuronal damage and memory loss. Sequential processing of APP(amyloid precursor protein) by β-/γ-secetase is required for Aβ production and the γ-secretase activity is the rate limiting step of the reaction. In fact pathogenic environment that regulates this enzyme complex is reported in AD patient case study. Thus, identification of genes that regulate its enzymatic activity is important for the treatment of Alzheimer's disease. **Methods:** We isolated novel ER localized γ-secretase activator (ELGA) by using cell-based functional screening system. **Results:** Overexpression of ELGA increased Aβ42 production and γ-secretase-mediated cleavage of APP-CTFβ in SH-SY5Y-APPswe cell. To confirm enzymatic activation effect of γ-secretase, we conducted in vitro cleavage assay and ELGA increased generation of APP-CTFγ. Interestingly, ELGA binds with Aph1 and PS1. In iodixanol gradient fractionation, subcellular localization of Aph1 and PS1-NTF seems to shift from ER to Golgi by ELGA. In addition, ELGA seems not to affect notch cleavage. **Conclusions:** These results suggest thatγELGA may stimulate γ-secretase assembly to increase the generation of Aβ.

3.1483 Lipid raft-dependent endocytosis: a new route for hepcidin-mediated regulation of ferroportin in macrophages

Auriac, A., Willemetz, A. and Canonne-Hergaux, F.
*Maemaatologica, **95(8)**, 1269-1277 (2010)*

Background: Expression of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and is decreased by hepcidin. We previously showed that ferroportin is present in specific cell surface domains suggestive of lipid rafts. Herein, we have clarified the localization of ferroportin in macrophage membranes and tested whether raft-mediated endocytosis plays a role in hepcidin activity.
Design and Methods: Raft/detergent-resistant membranes from murine bone marrow-derived macrophages and J774a1 cells were analyzed by Western blotting. The effect of lipid raft- or clathrin-dependent endocytosis inhibitors was studied on hepcidin activity. For this purpose, after treatment, ferroportin expression was analyzed by fluorescence microscopy, Western blotting of total protein extracts or plasma membrane protein samples, and by quantitative immunofluorescence assay (In-Cell-Western).
Results: Macrophage ferroportin was mostly detected in detergent-resistant membranes containing raft markers (caveolin 1, flotillin 1). Interestingly, iron overload strongly increased the presence of ferroportin in the lightest raft fraction. Moreover, lipid raft breakdown by cholesterol sequestration (filipin) or depletion (methyl-beta-cyclodextrin) decreased hepcidin activity on macrophage ferroportin. Cell surface biotinylation and immunofluorescence studies indicated that the process of both hepcidin mediated endocytosis and degradation of ferroportin were affected. By contrast, the inhibition of clathrin dependent endocytosis did not interfere with hepcidin effect.
Conclusions: Macrophage ferroportin is present in lipid rafts which contribute to hepcidin activity. These observations reveal the existence of a new cellular pathway in hepcidin mediated degradation of ferroportin and open a new area of investigation in mammalian iron homeostasis.

3.1484 Pgrmc1 (Progesterone Receptor Membrane Component 1) Associates with Epidermal Growth Factor Receptor and Regulates Erlotinib Sensitivity

Ahmed, I.S., Rohe, H.J., Twist, K.E. and Craven, R.J.
*J. Biol. Chem., **285(32)**, 24775-24782 (2010)*

Tumorigenesis requires the concerted action of multiple pathways, including pathways that stimulate proliferation and metabolism. Epidermal growth factor receptor (EGFR) is a transmembrane receptor-tyrosine kinase that is associated with cancer progression, and the EGFR inhibitors erlotinib/tarceva and tyrphostin/AG-1478 are potent anti-cancer therapeutics. Pgrmc1 (progesterone receptor membrane component 1) is a cytochrome b_5-related protein that is up-regulated in tumors and promotes cancer growth. Pgrmc1 and its homologues have been implicated in cell signaling, and we show here that Pgrmc1 increases susceptibility to AG-1478 and erlotinib, increases plasma membrane EGFR levels, and co-precipitates with EGFR. Pgrmc1 co-localizes with EGFR in cytoplasmic vesicles and co-fractionates with EGFR in high density microsomes. The findings have therapeutic potential because a Pgrmc1 small molecule ligand, which inhibits growth in a variety of cancer cell types, de-stabilized EGFR in multiple tumor cell lines. EGFR is one of the most potent receptor-tyrosine kinases driving tumorigenesis, and our data support a role for Pgrmc1 in promoting several cancer phenotypes at least in part by binding EGFR and stabilizing plasma membrane pools of the receptor.

3.1485 The fast-mobility isoform of mouse Mcl-1 is a mitochondrial matrix-localized protein with attenuated anti-apoptotic activity
Huang, C-R. and Yang-Yen, H-F.
*FEBS Lett., **584**, 3323-3330 (2010)*

The full-length pro-survival protein Mcl-1 predominantly resides on the outer membrane of mitochondria. Here, we identified a mitochondrial matrix-localized isoform of Mcl-1 that lacks 33 amino acid residues at the N-terminus which serve both as a mitochondrial targeting and processing signal. Ectopically-expressed Mcl-1 without the N-terminal 33 residues failed to enter the mitochondrial matrix but retained wt-like activities both for interaction with BH3-only proteins and anti-apoptosis. In contrast, the mitochondrial matrix-localized isoform failed to interact with BH3-only proteins and manifested an attenuated anti-apoptotic activity. This study reveals that import of Mcl-1 into the mitochondrial matrix results in the attenuation of Mcl-1's anti-apoptotic function.

3.1486 Mitochondrial DNA sequences are present inside nuclear DNA in rat tissues and increase with age
Caro, P., Gomez, J., Arduini, A., Gonzales-Sanchez, M., Gonzales-Garcia, M., Borras, C., Vina, J., Puertas, M.J:, Sastre, J. and Barja, G.
*Mitochondrion, **10**, 479-486 (2010)*

Mitochondrial DNA (mtDNA) mutations increase with age. However, the number of cells with predominantly mutated mtDNA is small in old animals. Here a new hypothesis is proposed: mtDNA fragments may insert into nuclear DNA contributing to aging and related diseases by alterations in the nucleus. Real-time PCR quantification shows that sequences of cytochrome oxidase III and 16S rRNA from mtDNA are present in highly purified nuclei from liver and brain in young and old rats. The sequences of these insertions revealed that they contain single nucleotide polymorphisms identical to those present in mtDNA of the same animal. Interestingly, the amount of mitochondrial sequences in nuclear DNA increases with age in both tissues. In situ hybridization of mtDNA to nuclear DNA confirms the presence of mtDNA sequences inside nuclear DNA in rat hepatocytes. Bone marrow metaphase cells from both young and old rats show mtDNA at centromeric regions in 20 out of the $2n = 40$ chromosomes. Consequently, mitochondria can be a major trigger of aging but the final target could also be the nucleus.

3.1487 Modulation of intracellular trafficking by TGFB and rhoa
Ribe, D. and Stenbeck, G.
*Bone, **47**, Suppl. 1, S130-S131 (2010)*

Bone diseases such as osteoarthritis and osteoporosis are characterised by defects in transforming growth factor beta (TGFβ) signalling and matrix protein deposition. We are interested in the immediate early effects of TGFβ in matrix secretion. We are studying the trafficking and secretion of the matrix proteins osteonectin (ON) and osteopontin (OP) in osteoblasts using total internal reflection fluorescence microscopy (TIRFM) and membrane fractionation.
ROS 17/2.8 cells were transfected with a plasmid for expression of green fluorescent protein (GFP) – tagged ON and examined by TIRFM. Dynamics of ON-containing vesicles were studied by taking TIRFM images of individual cells at 0.3 s intervals for 30 s. Vesicles were identified and their trajectories tracked in ImageJ using the Particle Tracker plugin. Membrane fractions of non-transfected ROS 17/2.8 cells were separated by ultracentrifugation on an Optiprep density gradient and examined by Western blotting.
In unstimulated cells, a minority of ON-containing vesicles displayed sustained fast movement along their trajectories (150 nm in 3 s). Treatment of cells with 25 nM TGFβ increased the number of these faster vesicles by 34% within 10 min, an effect that was sustained 20 min after addition of TGFβ. Vesicle motility reverted to the unstimulated state within 60 min of TGFβ treatment.
Pre-treatment of cells for 60 min with 2.5 μM of the ROCK inhibitor Y-27632 reduced the mean vesicle speed and displacement by 28% and 43% respectively. Vesicle speed, displacement and the number of fast vesicles did not recover with addition of 25 nM TGFβ to Y-27632 –treated cells. Treatment with 10 μM nocodazole for 20 min completely inhibited vesicle motility, indicating that the microtubule network is essential for fidelity of membrane trafficking.
Western blotting of membrane fractions separated on a density gradient showed that blocking the endogenous TGFβ signal with the monoclonal TGFβ antibody mAb240 (1 μg/ml) for 60 min caused a shift in the distribution of OP from a vesicular localisation towards fractions rich in Golgi markers.
These studies show that TGFβ has immediate effects on trafficking of the secreted proteins ON and OP in osteoblasts, which are modulated by the Rho signalling cascade. These immediate early events in TGFβ

signalling may play a role not only in matrix protein secretion but also in the propagation of further signals that lead to proliferation and aberrant matrix deposition if uncontrolled.

3.1488 Sortilin Facilitates Signaling of Ciliary Neurotrophic Factor and Related Helical Type 1 Cytokines Targeting the gp130/Leukemia Inhibitory Factor Receptor β Heterodimer
Vejby Larsen, J., Hansen, M., Møller, B., Madsen, P., Scheller, J., Nielsen, M. and Munck Pedersen, C.
Mol. Cell. Biol., ***30(17)***, *4175-4187 (2010)*

Sortilin is a member of the Vps10p domain family of neuropeptide and neurotrophin binding neuronal receptors. The family members interact with and partly share a variety of ligands and partake in intracellular sorting and protein transport as well as in transmembrane signal transduction. Thus, sortilin mediates the transport of both neurotensin and nerve growth factor and interacts with their respective receptors to facilitate ligand-induced signaling. Here we report that ciliary neurotrophic factor (CNTF), and related ligands targeting the established CNTF receptor α, binds to sortilin with high affinity. We find that sortilin may have at least two functions: one is to provide rapid endocytosis and the removal of CNTF, something which is not provided by CNTF receptor α, and the other is to facilitate CNTF signaling through the gp130/leukemia inhibitory factor (LIF) receptor β heterodimeric complex. Interestingly, the latter function is independent of both the CNTF receptor α and ligand binding to sortilin but appears to implicate a direct interaction with LIF receptor β. Thus, sortilin facilitates the signaling of all helical type 1 cytokines, which engage the gp130/LIF receptor β complex.

3.1489 Subcellular organelle lipidomics in TLR-4-activated macrophages
Andreyev, A.Y., Fahy, E., Guan, Z., Kelly, S., Li, X., McDonald, J.G., Milne, S., Myers, D., Park, H., Ryan, A., Thompson, B.M., Wang, E., Zhao, Y., Brown, H.A., Merrill, A.H., Raetz, C.R.H., Russell, D.W., Subramaniam, S. and Dennis, E.A.
J. Lipid Res., ***51***, *2785-2797 (2010)*

Lipids orchestrate biological processes by acting remotely as signaling molecules or locally as membrane components that modulate protein function. Detailed insight into lipid function requires knowledge of the subcellular localization of individual lipids. We report an analysis of the subcellular lipidome of the mammalian macrophage, a cell type that plays key roles in inflammation, immune responses, and phagocytosis. Nuclei, mitochondria, endoplasmic reticulum (ER), plasmalemma, and cytoplasm were isolated from RAW 264.7 macrophages in basal and activated states. Subsequent lipidomic analyses of major membrane lipid categories identified 229 individual/isobaric species, including 163 glycerophospholipids, 48 sphingolipids, 13 sterols, and 5 prenols. Major subcellular compartments exhibited substantially divergent glycerophospholipid profiles. Activation of macrophages by the Toll-like receptor 4-specific lipopolysaccharide Kdo_2-lipid A caused significant remodeling of the subcellular lipidome. Some changes in lipid composition occurred in all compartments (e.g., increases in the levels of ceramides and the cholesterol precursors desmosterol and lanosterol). Other changes were manifest in specific organelles. For example, oxidized sterols increased and unsaturated cardiolipins decreased in mitochondria, whereas unsaturated ether-linked phosphatidylethanolamines decreased in the ER. We speculate that these changes may reflect mitochondrial oxidative stress and the release of arachidonic acid from the ER in response to cell activation.

3.1490 The ARMS/Kidins220 scaffold protein modulates synaptic transmission
Arevalo, J.C., Wu, S.H., Takahashi, T., Zhang, H., Yu, T., Yano, H., Milner, T.A., Tessorollo, L., Ninan, I., Arancio, O. and Chao, M.V.
Mol. Cell. Neurosci., ***45***, *92-100 (2010)*

Activity-dependent changes of synaptic connections are facilitated by a variety of scaffold proteins, including PSD-95, Shank, SAP97 and GRIP, which serve to organize ion channels, receptors and enzymatic activities and to coordinate the actin cytoskeleton. The abundance of these scaffold proteins raises questions about the functional specificity of action of each protein. Here we report that basal synaptic transmission is regulated in an unexpected manner by the ankyrin repeat-rich membrane-spanning (ARMS/Kidins220) scaffold protein. In particular, decreases in the levels of ARMS/Kidins220 *in vivo* led to an increase in basal synaptic transmission in the hippocampus, without affecting paired pulse facilitation. One explanation to account for the effects of ARMS/Kidins220 is an interaction with the AMPA receptor subunit, GluA1, which could be observed after immunoprecipitation. Importantly, shRNA and cell surface biotinylation experiments indicate that ARMS/Kidins220 levels have an impact on GluA1 phosphorylation and localization. Moreover, ARMS/Kidins220 is a negative regulator of AMPAR function, which was

confirmed by inward rectification assays. These results provide evidence that modulation of ARMS/Kidins220 levels can regulate basal synaptic strength in a specific manner in hippocampal neurons.

3.1491 CHL1 Is a Selective Organizer of the Presynaptic Machinery Chaperoning the SNARE
Andreyeva, A., Leshchyn'ska, I., Knepper, M., Betzel, C., Redecke, L., Sytnyk, V. and Schachner, M.
*PloSOne, **5(8)**, e12018 (2010)*

Proteins constituting the presynaptic machinery of vesicle release undergo substantial conformational changes during the process of exocytosis. While changes in the conformation make proteins vulnerable to aggregation and degradation, little is known about synaptic chaperones which counteract these processes. We show that the cell adhesion molecule CHL1 directly interacts with and regulates the activity of the synaptic chaperones Hsc70, CSP and αSGT. CHL1, Hsc70, CSP and αSGT form predominantly CHL1/Hsc70/αSGT and CHL1/CSP complexes in synapses. Among the various complexes formed by CHL1, Hsc70, CSP and αSGT, SNAP25 and VAMP2 induce chaperone activity only in CHL1/Hsc70/αSGT and CHL1/CSP complexes, respectively, indicating a remarkable selectivity of a presynaptic chaperone activity for proteins of the exocytotic machinery. In mice with genetic ablation of CHL1, chaperone activity in synapses is reduced and the machinery for synaptic vesicle exocytosis and, in particular, the SNARE complex is unable to sustain prolonged synaptic activity. Thus, we reveal a novel role for a cell adhesion molecule in selective activation of the presynaptic chaperone machinery.

3.1492 L1 syndrome mutations impair neuronal L1 function at different levels by divergent mechanisms
Schäfer, M.K.E., Nam, Y-C., Moumen, A., Keglowich, L., Bouche, E., Küffner, M., Bock, H.H., Rathjem, F.G., Raoulo, C. and Frotscher, M.
*Neurobiology of Disease, **40**, 222-237 (2010)*

Mutations in the human L1CAM gene cause neurodevelopmental disorders collectively referred to as L1 syndrome. Here, we investigated cellular pathomechanisms underlying two L1 syndrome mutations, R184Q and W1036L. We demonstrate that these mutations cause partial endoplasmic reticulum (ER) retention of L1, reduce L1 cell surface expression, but do not induce ER stress in neuronal NSC-34 cells. We provide evidence that surface trafficking of mutated L1 is affected by defective sorting to ER exit sites and attenuated ER export. However, in differentiated neuronal cultures and long-term cultured hippocampal slices, the L1-R184Q protein is restricted to cell bodies, whereas L1-W1036L also aberrantly localizes to dendrites. These trafficking defects preclude axonal targeting of L1, thereby affecting L1-mediated axon growth and arborization. Our results indicate that L1 syndrome mutations impair neuronal L1 function at different levels, firstly by attenuating ER export and secondly by interfering with polarized neuronal trafficking.

3.1493 Attenuation of the hypoxia-induced protein kinase Cδ interaction with the 'd' subunit of F1Fo-ATP synthase in neonatal cardiac myocytes: implications for energy preservation and survival
Nguyen, T.T., Ogbi, M., Yu, Q. and Johnson, J.A.
*Biochem. J., **429**, 335-345 (2010)*

The F_1F_o-ATP synthase provides most of the heart's energy, yet events that alter its function during injury are poorly understood. Recently, we described a potent inhibitory effect on F_1F_o-ATP synthase function mediated by the interaction of PKCδ (protein kinase Cδ) with dF_1F_o ('d' subunit of the F_1F_o-ATPase/ATP synthase). We have now developed novel peptide modulators which facilitate or inhibit the PKCδ–dF_1F_o interaction. These peptides include HIV-Tat (transactivator of transcription) protein transduction and mammalian mitochondrial-targeting sequences. Pre-incubation of NCMs (neonatal cardiac myocyte) with 10 nM extracellular concentrations of the mitochondrial-targeted PKCδ–dF_1F_o interaction inhibitor decreased Hx (hypoxia)-induced co-IP (co-immunoprecipitation) of PKCδ with dF_1F_o by 40±9%, abolished Hx-induced inhibition of F_1F_o-ATPase activity, attenuated Hx-induced losses in F_1F_o-derived ATP and protected against Hx- and reperfusion-induced cell death. A scrambled-sequence (inactive) peptide, which contained HIV-Tat and mitochondrial-targeting sequences, was without effect. In contrast, the cell-permeant mitochondrial-targeted PKCδ–dF_1F_o facilitator peptide, which we have shown previously to induce the PKCδ–dF_1F_o co-IP, was found to inhibit F_1F_o-ATPase activity to an extent similar to that caused by Hx alone. The PKCδ–dF_1F_o facilitator peptide also decreased ATP levels by 72±18% under hypoxic conditions in the presence of glycolytic inhibition. None of the PKCδ–dF_1F_o modulatory

peptides altered the inner mitochondrial membrane potential. Our studies provide the first evidence that disruption of the PKCδ–dF_1F_o interaction using cell-permeant mitochondrial-targeted peptides attenuates cardiac injury resulting from prolonged oxygen deprivation.

3.1494 Calcium- and polyphosphate-containing acidic granules of sea urchin eggs are similar to acidocalcisomes, but are not the targets for NAADP
Ramos, I.B., Miranda, K., Pace, D.A., Verbist, K.C., Lins, F-Y., Zhang, Y., Oldfield, E., Machado, E.A., De Souza, W. and Docompo, R.
Biochem. J., ***429***, *485-495 (2010)*

Acidocalcisomes are acidic calcium-storage compartments described from bacteria to humans and characterized by their high content in poly P (polyphosphate), a linear polymer of many tens to hundreds of P_i residues linked by high-energy phosphoanhydride bonds. In the present paper we report that millimolar levels of short-chain poly P (in terms of P_i residues) and inorganic PP_i are present in sea urchin extracts as detected using ^{31}P-NMR, enzymatic determinations and agarose gel electrophoresis. Poly P was localized to granules randomly distributed in the sea urchin eggs, as shown by labelling with the poly-P-binding domain of *Escherichia coli* exopolyphosphatase. These granules were enriched using iodixanol centrifugation and shown to be acidic and to contain poly P, as determined by Acridine Orange and DAPI (4′,6′-diamidino-2-phenylindole) staining respectively. These granules also contained large amounts of calcium, sodium, magnesium, potassium and zinc, as detected by X-ray microanalysis, and bafilomycin A_1-sensitive ATPase, pyrophosphatase and exopolyphosphatase activities, as well as Ca^{2+}/H^+ and Na^+/H^+ exchange activities, being therefore similar to acidocalcisomes described in other organisms. Calcium release from these granules induced by nigericin was associated with poly P hydrolysis. Although NAADP (nicotinic acid–adenine dinucleotide phosphate) released calcium from the granule fraction, this activity was not significantly enriched as compared with the NAADP-stimulated calcium release from homogenates and was not accompanied by poly P hydrolysis. GPN (glycyl-L-phenylalanine-naphthylamide) released calcium when added to sea urchin homogenates, but was unable to release calcium from acidocalcisome-enriched fractions, suggesting that these acidic stores are not the targets for NAADP.

3.1495 Dephosphorylation of F-BAR Protein Cdc15 Modulates Its Conformation and Stimulates Its Scaffolding Activity at the Cell Division Site
Roberts-Galbraith, R.H., Ohi, M.D., Ballif, B.A., Chen, J-S., McLeod, I., McDonald, W.H., Gygi, S.P., Yates III, J.R. and Gould, K.L.
Mol. Cell, ***39***, *86-99 (2010)*

Cytokinesis in *Schizosaccharomyces pombe* requires the function of Cdc15, the founding member of the *pombe cdc15* homology (PCH) family of proteins. As an early, abundant contractile ring component with multiple binding partners, Cdc15 plays a key role in organizing the ring. We demonstrate that Cdc15 phosphorylation at many sites generates a closed conformation, inhibits Cdc15 assembly at the division site in interphase, and precludes interaction of Cdc15 with its binding partners. Cdc15 dephosphorylation induces an open conformation, oligomerization, and scaffolding activity during mitosis. Cdc15 mutants with reduced phosphorylation precociously appear at the division site in filament-like structures and display increased association with protein partners and the membrane. Our results indicate that Cdc15 phosphoregulation impels both assembly and disassembly of the contractile apparatus and suggest a regulatory strategy that PCH family and BAR superfamily members might broadly employ to achieve temporal specificity in their roles as linkers between membrane and cytoskeleton.

3.1496 Secreted Monocytic miR-150 Enhances Targeted Endothelial Cell Migration
Zhang, Y. et al
Mol. Cell, ***39***, *133-144 (2010)*

MicroRNAs (miRNAs) are a class of noncoding RNAs that regulate target gene expression at the posttranscriptional level. Here, we report that secreted miRNAs can serve as signaling molecules mediating intercellular communication. In human blood cells and cultured THP-1 cells, miR-150 was selectively packaged into microvesicles (MVs) and actively secreted. THP-1-derived MVs can enter and deliver miR-150 into human HMEC-1 cells, and elevated exogenous miR-150 effectively reduced c-Myb expression and enhanced cell migration in HMEC-1 cells. In vivo studies confirmed that intravenous injection of THP-1 MVs significantly increased the level of miR-150 in mouse blood vessels. MVs

isolated from the plasma of patients with atherosclerosis contained higher levels of miR-150, and they more effectively promoted HMEC-1 cell migration than MVs from healthy donors. These results demonstrate that cells can secrete miRNAs and deliver them into recipient cells where the exogenous miRNAs can regulate target gene expression and recipient cell function.

3.1497 SH3TC2, a protein mutant in Charcot–Marie–Tooth neuropathy, links peripheral nerve myelination to endosomal recycling

Stendel, C., Roos, A., Kleine, H., Arnoud, E., Özcelik, M., Sidiropoulos, P.N.M., Zenker, J., Schhüpfer, F., Lehmann, U., Sobota, R.M., Litchfield, D.W., Lüscher, B., Chrast, R., Suter, U. and Senderek, J.

Patients with Charcot–Marie–Tooth neuropathy and gene targeting in mice revealed an essential role for the *SH3TC2* gene in peripheral nerve myelination. *SH3TC2* expression is restricted to Schwann cells in the peripheral nervous system, and the gene product, SH3TC2, localizes to the perinuclear recycling compartment. Here, we show that SH3TC2 interacts with the small guanosine triphosphatase Rab11, which is known to regulate the recycling of internalized membranes and receptors back to the cell surface. Results of protein binding studies and transferrin receptor trafficking are in line with a role of SH3TC2 as a Rab11 effector molecule. Consistent with a function of Rab11 in Schwann cell myelination, *SH3TC2* mutations that cause neuropathy disrupt the SH3TC2/Rab11 interaction, and forced expression of dominant negative Rab11 strongly impairs myelin formation *in vitro*. Our data indicate that the SH3TC2/Rab11 interaction is relevant for peripheral nerve pathophysiology and place endosomal recycling on the list of cellular mechanisms involved in Schwann cell myelination.

3.1498 PERK (EIF2AK3) Regulates Proinsulin Trafficking and Quality Control in the Secretory Pathway

Gupta, S., McGrath, B. and Cavener, D.R.
Diabetes, ***59****, 1937-1947 (2010)*

OBJECTIVE Loss-of-function mutations in *Perk* (EIF2AK3) result in permanent neonatal diabetes in humans (Wolcott-Rallison Syndrome) and mice. Previously, we found that diabetes associated with *Perk* deficiency resulted from insufficient proliferation of β-cells and from defects in insulin secretion. A substantial fraction of PERK-deficient β-cells display a highly abnormal cellular phenotype characterized by grossly distended endoplasmic reticulum (ER) and retention of proinsulin. We investigated over synthesis, lack of ER-associated degradation (ERAD), and defects in ER to Golgi trafficking as possible causes.
RESEARCH DESIGN AND METHODS ER functions of PERK were investigated in cell culture and mice in which *Perk* was impaired or gene dosage modulated. The $Ins2^{+/Akita}$ mutant mice were used as a model system to test the role of PERK in ERAD.
RESULTS We report that loss of *Perk* function does not lead to uncontrolled protein synthesis but impaired ER-to-Golgi anterograde trafficking, retrotranslocation from the ER to the cytoplasm, and proteasomal degradation. PERK was also shown to be required to maintain the integrity of the ER and Golgi and processing of ATF6. Moreover, decreasing *Perk* dosage surprisingly ameliorates the progression of the *Akita* mutants toward diabetes.
CONCLUSIONS PERK is a positive regulator of ERAD and proteasomal activity. Reducing PERK activity ameliorates the progression of diabetes in the Akita mouse, whereas increasing PERK dosage hastens its progression. We speculate that PERK acts as a metabolic sensor in the insulin-secreting β-cells to modulate the trafficking and quality control of proinsulin in the ER relative to the physiological demands for circulating insulin.

3.1499 Cholesterol depletion alters amplitude and pharmacology of vascular calcium-activated chloride channels

Sones, W.R., Davis, A.J., Leblanc, N. and Greenwood, A.
Cardiovasc. Res., ***87****, 476-484 (2010)*

Aims Calcium-activated chloride channels (CACCs) share common pharmacological properties with Kcnma1-encoded large conductance K^+ channels (BK_{Ca} or $K_{Ca}1.1$) and it has been suggested that they may co-exist in a macromolecular complex. As $K_{Ca}1.1$ channels are known to localize to cholesterol and caveolin-rich lipid rafts (caveolae), the present study investigated whether Ca^{2+}-sensitive Cl^- currents in vascular myocytes were affected by the cholesterol depleting agent methyl-β-cyclodextrin (M-βCD).
Methods and results Calcium-activated chloride and potassium currents were recorded from single murine portal vein myocytes in whole cell voltage clamp. Western blot was undertaken following sucrose gradient ultracentrifugation using protein lysates from whole portal veins. Ca^{2+}-activated Cl^- currents were

augmented by 3 mg mL^{-1} M-βCD with a rapid time course ($t_{0.5}$ = 1.8 min). M-βCD had no effect on the bi-modal response to niflumic acid or anthracene-9-carboxylate but completely removed the inhibitory effects of the $K_{Ca}1.1$ blockers, paxilline and tamoxifen, as well as the stimulatory effect of the $K_{Ca}1.1$ activator NS1619. Discontinuous sucrose density gradients followed by western blot analysis revealed that the position of lipid raft markers caveolin and flotillin-2 was altered by 15 min application of 3 mg mL^{-1} M-βCD. The position of $K_{Ca}1.1$ and the newly identified candidate for CACCs, TMEM16A, was also affected by M-βCD.

3.1500 Do Caveolae Have a Role in the Fidelity and Dynamics of Receptor Activation of G-protein-gated Inwardly Rectifying Potassium Channels?
Schwarzer, S., Nobles, M. and Tinker, A.
J. Biol. Chem., ***285(36)***, *27817-27826 (2010)*

In atrial and nodal cardiac myocytes, M2 muscarinic receptors activate inhibitory G-proteins ($G_{i/o}$), which in turn stimulate G-protein-gated inwardly rectifying K^+ channels through direct binding of the Gβγ subunit. Despite also releasing Gβγ, G_s-coupled receptors such as the β-adrenergic receptor are not able to prominently activate this current. An appealing hypothesis would be if components were sequestered in membrane domains such as caveolae/rafts. Using biochemical fractionation followed by Western blotting and/or radioligand binding experiments, we examined the distribution of the components in stable HEK293 and HL-1 cells, which natively express the transduction cascade. The channel, M2 muscarinic, and A1 adenosine receptors were located in noncaveolar/nonraft fractions. $G_i\alpha_{1/2}$ was enriched in both caveolar/raft and noncaveolar/nonraft fractions. In contrast, $G_s\alpha$ was only enriched in caveolar/raft fractions. We constructed YFP-tagged caveolin-2 (YFP-Cav2) and chimeras with the M2 (M2-YFP-Cav2) and A1 (A1-YFP-Cav2) receptors. Analysis of gradient fractions showed that these receptor chimeras were now localized to caveolae-enriched fractions. Microscopy showed that M2-YFP and A1-YFP had a diffuse homogenous membrane signal. YFP-Cav2, M2-YFP-Cav2, and A1-YFP-Cav2 revealed a more punctuate pattern. Finally, we looked at the consequences for signaling. Activation via M2-YFP-Cav2 or A1-YFP-Cav2 revealed substantially slower kinetics compared with M2-YFP or A1-YFP and was reversed by the addition of methyl-β-cyclodextrin. Thus the localization of the channel signal transduction cascade in non-cholesterol rich domains substantially enhances the speed of signaling. The presence of $G_s\alpha$ solely in caveolae may account for signaling selectivity between $G_{i/o}$ and G_s-coupled receptors.

3.1501 Neogenin Regulation of BMP-Induced Canonical Smad Signaling and Endochondral Bone Formation
Zhou, Z., Xie, J., Lee, D., Liu, Y., Jung, J., Zhou, L., Xiong, S., Mei, L. and Xiong, W-C.
Developmental Cell, ***19***, *90-102 (2010)*

Neogenin has been identified as a receptor for the neuronal axon guidance cues netrins and RGMs (repulsive guidance molecules). Here we provide evidence for neogenin in regulating endochondral bone development and BMP (bone morphogenetic protein) signaling. Neogenin-deficient mice were impaired in digit/limb development and endochondral ossification. BMP2 induction of Smad1/5/8 phosphorylation and Runx2 expression, but not noncanonical p38 MAPK activation, was reduced in chondrocytes from neogenin mutant mice. BMP receptor association with membrane microdomains, which is necessary for BMP signaling to Smad, but not p38 MAPK, was diminished in neogenin-deficient chondrocytes. Furthermore, RGMs appear to mediate neogenin interaction with BMP receptors in chondrocytes. Taken together, our results indicate that neogenin promotes chondrogenesis in vitro and in vivo, revealing an unexpected mechanism underlying neogenin regulation of BMP signaling.

3.1502 Suppression of the novel ER protein Maxer by mutant ataxin-1 in Bergman glia contributes to non-cell-autonomous toxicity
Shiwaku, H., Yoshimura, N., Tamura, T., Sone, M., Ogisgima, S., Watase, K., Tagawe, K. and Okazawa, H.
EMBO J., ***29***, *2446-2460 (2010)*

Non-cell-autonomous effect of mutant proteins expressed in glia has been implicated in several neurodegenerative disorders, whereas molecules mediating the toxicity are currently not known. We identified a novel molecule named multiple α-helix protein located at ER (Maxer) downregulated by mutant ataxin-1 (Atx1) in Bergmann glia. Maxer is an endoplasmic reticulum (ER) membrane protein interacting with CDK5RAP3. Maxer anchors CDK5RAP3 to the ER and inhibits its function of Cyclin D1 transcription repression in the nucleus. The loss of Maxer eventually induces cell accumulation at G1

phase. It was also shown that mutant Atx1 represses Maxer and inhibits proliferation of Bergmann glia in vitro. Consistently, Bergmann glia are reduced in the cerebellum of mutant Atx1 knockin mice before onset. Glutamate-aspartate transporter reduction in Bergmann glia by mutant Atx1 and vulnerability of Purkinje cell to glutamate are both strengthened by Maxer knockdown in Bergmann glia, whereas Maxer overexpression rescues them. Collectively, these results suggest that the reduction of Maxer mediates functional deficiency of Bergmann glia, and might contribute to the non-cell-autonomous pathology of SCA1.

3.1503 Localization and trafficking of endogenous anterior pharynx-defective 1, a component of Alzheimer's disease related γ-secretase

Sanjo, N., Katayama, T., Hasegawa, H., Jin, H., Duthie, M., Mount, H.T.J., Mizusawa, H., St George-Hyslop, P and Fraser, P.E.
Neurosci. Lett., ***483***, *53-56 (2010)*

Anterior pharynx-defective 1 (Aph-1) is a multi-spanning membrane protein and an integral component of the high molecular weight γ-secretase complex that also contains presenilin, nicastrin, and Pen-2. In order to clarify the existence of an endogenous fragment of Aph-1 and dissect the localization and processing of endogenous Aph-1 proteins, we examined cell lines and primary cell cultures with our own carboxyl terminal-specific antibodies for Aph-1aL. Fractionation and immunofluorescence studies indicated that the endogenous full-length Aph-1aL isoform localizes primarily to the endoplasmic reticulum as well as Golgi intermediate compartment, but small amount of it was detected at Golgi apparatus where most of its carboxyl terminal domain fragment existed. In primary neuronal and glial cultures, Aph-1aL was present in the neurites and glial cell processes. Endogenous Aph-1a and its proteolytic fragment have unique properties for cleavage control that may have implications for γ-secretase regulation and intracellular distribution.

3.1504 α2β1 integrin controls association of Rac with the membrane and triggers quiescence of endothelial cells

Cailleteau, L., Estrach, S., Thyss, R., Boyer, L., Doye, A., Domange, B., Johnsson, N., Rubinstein, E., Boucheix, C., Ebrahimian, T., Silvestre, J-S., Lemichez, E., Meneguzzi, G. and Mettouchi, A.
J. Cell Sci., ***123***, *2491-2501 (2010)*

Integrin receptors and their extracellular matrix ligands provide cues to cell proliferation, survival, differentiation and migration. Here, we show that α2β1 integrin, when ligated to the basement membrane component laminin-1, triggers a proliferation arrest in primary endothelial cells. Indeed, in the presence of strong growth signals supplied by growth factors and fibronectin, α2β1 engagement alters assembly of mature focal adhesions by α5β1 and leads to impairment of downstream signaling and cell-cycle arrest in the G1 phase. Although the capacity of α5β1 to signal for GTP loading of Rac is preserved, the joint engagement of α2β1 interferes with membrane anchorage of Rac. Adapting the 'split-ubiquitin' sensor to screen for membrane-proximal α2 integrin partners, we identified the CD9 tetraspanin and further establish its requirement for destabilization of focal adhesions, control of Rac subcellular localization and growth arrest induced by α2β1 integrin. Altogether, our data establish that α2β1 integrin controls endothelial cell commitment towards quiescence by triggering a CD9-dependent dominant signaling.

3.1505 NCAM-Induced Neurite Outgrowth Depends on Binding of Calmodulin to NCAM and on Nuclear Import of NCAM and fak Fragments

Kleene, R., Mzoughi, M., Joshi, G., Kalus, I., Bormann, U., Schulze, C., Xiao, M-F., Dityatev, A. and Schachner, M.
J. Neurosci., ***30(32)***, *10784-10798 (2010)*

The neural cell adhesion molecule NCAM plays important functional roles not only during nervous system development, but also in the adult after injury and in synaptic plasticity. Homophilic binding of NCAM triggers intracellular signaling events resulting in cellular responses such as neurite outgrowth that require NCAM palmitoylation-dependent raft localization and activation of the nonreceptor tyrosine kinases fyn and fak. In this study, we show that stimulation of NCAM by a function-triggering NCAM antibody results in proteolytic processing of NCAM and fak. The C-terminal fragment of NCAM, consisting of the intracellular domain, the transmembrane domain, and a stub of the extracellular domain, and the N-terminal fragment of fak are imported into the nucleus. NCAM-stimulated fak activation, generation, and nuclear import of NCAM and fak fragments as well as neurite outgrowth are abolished by mutation of the calmodulin binding motif in the intracellular domain of NCAM that is responsible for the calcium-

dependent binding of calmodulin to NCAM. This mutation interferes neither with NCAM cell surface expression, palmitoylation, and raft localization nor with fyn activation. The way by which the transmembrane NCAM fragment reaches the nucleus in a calmodulin- and calcium-dependent manner is by endocytotic transport via the endoplasmic reticulum and the cytoplasm. The generation and nuclear import of NCAM and phosphorylated fak fragments resulting from NCAM stimulation may represent a signal pathway activating cellular responses in parallel or in association with classical kinase- and phosphorylation-dependent signaling cascades.

3.1506 Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association

Rogers, K.R., Kikawa, K.D., Mouradian, M., Hernandez, K., McKinnon, K., Ahwah, S.M. and Pardini, R.S.

*Carcinogenesis, **31(9)**, 1523-1530 (2010)*

Docosahexaenoic acid (DHA), a 22:6 n-3 polyunsaturated fatty acid, is the longest and most highly unsaturated fatty acid found in most membranes and has been shown to inhibit cancer cell growth in part by modifying cell signaling. In the current study, alterations to epidermal growth factor receptor (EGFR) signaling upon DHA supplementation are examined in A549 lung adenocarcinoma, WiDr colon carcinoma and MDA-MB-231 breast carcinoma cell lines. Interestingly, EGFR phosphorylation, most notably at the tyrosine 1068 residue, is dramatically upregulated, and EGFR association with the Sos1 guanine nucleotide exchange factor is concomitantly increased upon DHA supplementation. However, guanosine triphosphate-bound Ras and phosphorylated extracellular signal-regulated kinase (Erk)1/2 are paradoxically downregulated in the same treatments. Previous reports have noted changes in membrane microdomains upon DHA supplementation, and our findings confirmed that EGFR, but not Ras, is excluded from caveolin-rich lipid raft fractions in DHA-treated cells, resulting in a decreased association of Ras with Sos1 and the subsequent downregulation of Erk signaling. Xenografts of the A549 cell line implanted in athymic mice fed a control high-fat diet or a diet high in DHA confirmed our in vitro data. These results demonstrate for the first time a functional consequence of decreased EGFR protein in lipid raft microdomains as a result of DHA treatment in three different cancer models. In addition, we report the ability of DHA to enhance the efficacy of EGFR inhibitors on anchorage-independent cell growth (soft agar), providing evidence for the potential development of enhanced combination therapies.

3.1507 Effects of cholesterol on CCK-1 receptors and caveolin-3 proteins recycling in human gallbladder muscle

Cong, P., Pricolo, V., Biancani, P. and Behar, J.

*Am. J. Physiol. Gastrointest. Liver Physiol., **299**, G742-G750 (2010)*

The contraction of gallbladders (GBs) with cholesterol stones is impaired due to high cholesterol concentrations in caveolae compared with GBs with pigment stones. The reduced contraction is caused by a lower cholecystokinin (CCK)-8 binding to CCK-1 receptors (CCK-1R) due to caveolar sequestration of receptors. We aimed to examine the mechanism of cholesterol-induced sequestration of receptors. Muscle cells from human and guinea pig GBs were studied. Antibodies were used to examine CCK-1R, antigens of early and recycling endosomes, and total (CAV-3) and phosphorylated caveolar-3 protein (pCAV-3) by Western blots. Contraction was measured in muscle cells transfected with CAV3 mRNA or clathrin heavy-chain small-interfering RNA (siRNA). CCK-1R returned back to the bulk plasma membrane (PM) 30 min after CCK-8 recycled by endosomes, peaking at 5 min in early endosomes and at 20 min in recycling endosomes. Pretreatment with cholesterol-rich liposomes inhibited the transfer of CCK-1R and of CAV-3 in the endosomes by blocking CAV-3 phosphorylation. 4-Amino-5-(4-chloro-phenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (inhibitor of tyrosine kinase) reproduced these effects by blocking pCAV-3 formation, increasing CAV-3 and CCK-1R sequestration in the caveolae and impairing CCK-8-induced contraction. CAV-3 siRNA reduced CAV-3 protein expression, decreased CCK-8-induced contraction, and accumulated CCK-1R in the caveolae. Abnormal concentrations of caveolar cholesterol had no effect on met-enkephalin that stimulates a δ-opioid receptor that internalizes through clathrin. We found that impaired muscle contraction in GBs with cholesterol stones is due to high caveolar levels of cholesterol that inhibits pCAV-3 generation. Caveolar cholesterol increases the caveolar sequestration of CAV-3 and CCK-1R caused by their reduced recycling to the PM.

3.1508 Hsp12 Is an Intrinsically Unstructured Stress Protein that Folds upon Membrane Association and Modulates Membrane Function

Welker, S., Rudolph, B., Frenzel, E., Hagn, F., Liebisch, G., Schmitz, G., Scheuring, J., Kerth, A., Blume, A., Weinkauf, S., Haslbeck, M., Kessler, H. and Buchner, J.
*Molecular Cell, **39**, 507-520 (2010)*

Hsp12 of *S. cerevisiae* is upregulated several 100-fold in response to stress. Our phenotypic analysis showed that this protein is important for survival of a variety of stress conditions, including high temperature. In the absence of Hsp12, we observed changes in cell morphology under stress conditions. Surprisingly, in the cell, Hsp12 exists both as a soluble cytosolic protein and associated to the plasma membrane. The in vitro analysis revealed that Hsp12, unlike all other Hsps studied so far, is completely unfolded; however, in the presence of certain lipids, it adopts a helical structure. The presence of Hsp12 does not alter the overall lipid composition of the plasma membrane but increases membrane stability.

3.1509 Misfolded Mutant SOD1 Directly Inhibits VDAC1 Conductance in a Mouse Model of Inherited ALS

Israelson, A., Arbel, N., Da Cruz, S., Lliweva, H., Yamanaka, K., Shoshan-Barmatz, V. and Cleveland, D.W.
*Cell, **67**, 575-587 (2010)*

Mutations in superoxide dismutase (SOD1) cause amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by loss of motor neurons. With conformation-specific antibodies, we now demonstrate that misfolded mutant SOD1 binds directly to the voltage-dependent anion channel (VDAC1), an integral membrane protein imbedded in the outer mitochondrial membrane. This interaction is found on isolated spinal cord mitochondria and can be reconstituted with purified components in vitro. ADP passage through the outer membrane is diminished in spinal mitochondria from mutant SOD1-expressing ALS rats. Direct binding of mutant SOD1 to VDAC1 inhibits conductance of individual channels when reconstituted in a lipid bilayer. Reduction of VDAC1 activity with targeted gene disruption is shown to diminish survival by accelerating onset of fatal paralysis in mice expressing the ALS-causing mutation $SOD1^{G37R}$. Taken together, our results establish a direct link between misfolded mutant SOD1 and mitochondrial dysfunction in this form of inherited ALS.

3.1510 Membrane Vesicles: A Common Feature in the Extracellular Matter of Cold-Adapted Antarctic Bacteria

Frias, A., Manresa, A., de Oliveira, E., Lopez-Inglisias, C. and Mercade, E.
*Microb. Ecol., **59**, 476-486 (2010)*

Many Gram-negative, cold-adapted bacteria from the Antarctic environment produce large amounts of extracellular matter, which has potential biotechnology applications. We examined the ultrastructure of extracellular matter from five Antarctic bacteria (*Shewanella livingstonensis* $NF22^T$, *Shewanella vesiculosa* $M7^T$, *Pseudoalteromonas* sp. M4.2, *Psychrobacter fozii* $NF23^T$, and *Marinobacter guineae* $M3B^T$) by transmission electron microscopy after high-pressure freezing and freeze substitution. All analyzed extracellular matter appeared as a netlike mesh composed of a capsular polymer around cells and large numbers of membrane vesicles (MVs), which have not yet been described for members of the genera *Psychrobacter* and *Marinobacter*. MVs showed the typical characteristics described for these structures, and seemed to be surrounded by the same capsular polymer as that found around the cells. The analysis of MV proteins from Antarctic strains by SDS-PAGE showed different banding profiles in MVs compared to the outer membrane, suggesting some kind of protein sorting during membrane vesicle formation. For the psychrotolerant bacterium, *S. livingstonensis* $NF22^T$, the growth temperature seemed to influence the amount and morphology of MVs. In an initial attempt to elucidate the functions of MVs for this psychrotolerant bacterium, we conducted a proteomic analysis on membrane vesicles from *S. livingstonensis* $NF22^T$ obtained at 4 and 18°C. At both temperatures, MVs were highly enriched in outer membrane proteins and periplasmic proteins related to nutrient processing and transport in Gram-negative bacteria suggesting that MVs could be related with nutrient sensing and bacterial survival. Differences were observed in the expression of some proteins depending on incubation temperature but further studies will be necessary to define their roles and implications in the survival of bacteria in the extreme Antarctic environment.

3.1511 **Dihydrosphingomyelin Impairs HIV-1 Infection by Rigidifying Liquid-Ordered Membrane Domains**
Vieira, C.R. et al
*Chemistry & Biology, **17**, 766-775 (2010)*

The lateral organization of lipids in cell membranes is thought to regulate numerous cell processes. Most studies focus on the coexistence of two fluid phases, the liquid crystalline (l_d) and the liquid-ordered (l_o); the putative presence of gel domains (s_o) is not usually taken into account. We show that in phospholipid:sphingolipid:cholesterol mixtures, in which sphingomyelin (SM) promoted fluid l_o domains, dihydrosphingomyelin (DHSM) tended to form rigid domains. Genetic and pharmacological blockade of the dihydroceramide desaturase (Des1), which replaced SM with DHSM in cultured cells, inhibited cell infection by replication-competent and -deficient HIV-1. Increased DHSM levels gave rise to more rigid membranes, resistant to the insertion of the gp41 fusion peptide, thus inhibiting viral-cell membrane fusion. These results clarify the function of dihydrosphingolipids in biological membranes and identify Des1 as a potential target in HIV-1 infection.

3.1512 **COPI-mediated retrograde trafficking from the Golgi to the ER regulates EGFR nuclear transport**
Wang, Y-N., Wang, H., Yamaguchi, H., Lee, H-J., Lee, H-H. and Hung, M-C.
*Biochem. Biophys. Res. Comm., **399**, 498-504 (2010)*

Emerging evidence indicates that cell surface receptors, such as the entire epidermal growth factor receptor (EGFR) family, have been shown to localize in the nucleus. A retrograde route from the Golgi to the endoplasmic reticulum (ER) is postulated to be involved in the EGFR trafficking to the nucleus; however, the molecular mechanism in this proposed model remains unexplored. Here, we demonstrate that membrane-embedded vesicular trafficking is involved in the nuclear transport of EGFR. Confocal immunofluorescence reveals that in response to EGF, a portion of EGFR redistributes to the Golgi and the ER, where its NH_2-terminus resides within the lumen of Golgi/ER and COOH-terminus is exposed to the cytoplasm. Blockage of the Golgi-to-ER retrograde trafficking by brefeldin A or dominant mutants of the small GTPase ADP-ribosylation factor, which both resulted in the disassembly of the coat protein complex I (COPI) coat to the Golgi, inhibit EGFR transport to the ER and the nucleus. We further find that EGF-dependent nuclear transport of EGFR is regulated by retrograde trafficking from the Golgi to the ER involving an association of EGFR with γ-COP, one of the subunits of the COPI coatomer. Our findings experimentally provide a comprehensive pathway that nuclear transport of EGFR is regulated by COPI-mediated vesicular trafficking from the Golgi to the ER, and may serve as a general mechanism in regulating the nuclear transport of other cell surface receptors.

3.1513 **Exosomes: Extracellular organelles important in intercellular communication**
Mathivanan, S., Ji, H. and Simpson, R.J.
*J. Proteomics, **73**, 1907-1920 (2010)*

In addition to intracellular organelles, eukaryotic cells also contain extracellular organelles that are released, or shed, into the microenvironment. These membranous extracellular organelles include exosomes, shedding microvesicles (SMVs) and apoptotic blebs (ABs), many of which exhibit pleiotropic biological functions. Because extracellular organelle terminology is often confounding, with many preparations reported in the literature being mixtures of extracellular vesicles, there is a growing need to clarify nomenclature and to improve purification strategies in order to discriminate the biochemical and functional activities of these moieties. Exosomes are formed by the inward budding of multivesicular bodies (MVBs) and are released from the cell into the microenvironment following the fusion of MVBs with the plasma membrane (PM). In this review we focus on various strategies for purifying exosomes and discuss their biophysical and biochemical properties. An update on proteomic analysis of exosomes from various cell types and body fluids is provided and host-cell specific proteomic signatures are also discussed. Because the ectodomain of ~ 42% of exosomal integral membrane proteins are also found in the secretome, these vesicles provide a potential source of serum-based membrane protein biomarkers that are reflective of the host cell. ExoCarta, an exosomal protein and RNA database (http://exocarta.ludwig.edu.au), is described.

3.1514 **Palmitoylation of CD36/FAT regulates the rate of its post-transcriptional processing in the endoplasmic reticulum**
Thorne, R.F., Ralston, K.J., de Bock, C.E., Nhaidat, N.M., Zhang, X.D., Boyd, A.W. and Burns, G.F.
*Biochim. Biophys. Acta, **1803**, 1298-1307 (2010)*

CD36/FAT is a transmembrane glycoprotein that functions in the cellular uptake of long-chain fatty acids and also as a scavenger receptor. As such it plays an important role in lipid homeostasis and, pathophysiologically, in the progression of type 2 diabetes and atherosclerosis. CD36 expression is tightly regulated at the levels of both transcription and translation. Here we show that its expression and location are also regulated post-translationally, by palmitoylation. Although palmitoylation of CD36 was not required for receptor maturation and cell surface expression, inhibition of palmitoylation either pharmacologically with cerulenin or by mutation of the relevant cysteines delayed processing at the ER and trafficking through the secretory pathway. The absence of palmitoylation also reduced the half life of the CD36 protein. Additionally, the CD36 palmitoylation mutant did not incorporate efficiently into lipid rafts, a site known to be required for its function of fatty acid uptake, and this reduced the efficiency of uptake of oxidized low density lipoprotein. These findings provide an added level of sophistication where translocation of CD36 to the plasma membrane may be physiologically regulated by palmitoylation.

3.1515 Accessibility of Cholesterol in Endoplasmic Reticulum Membranes and Activation of SREBP-2 Switch Abruptly at a Common Cholesterol Threshold
Sokolov, A. and Radhakrishnan, A.
J. Biol. Chem., ***285(38)***, *29480-29490 (2010)*

Recent studies have shown that cooperative interactions in endoplasmic reticulum (ER) membranes between Scap, cholesterol, and Insig result in switch-like control over activation of SREBP-2 transcription factors. This allows cells to rapidly adjust rates of cholesterol synthesis and uptake in response to even slight deviations from physiological set-point levels, thereby ensuring cholesterol homeostasis. In the present study we directly probe for the accessibility of cholesterol in purified ER membranes. Using a soluble cholesterol-binding bacterial toxin, perfringolysin O, we show that cholesterol accessibility increases abruptly at ~ 5 mol % ER cholesterol, the same concentration at which SREBP-2 activation is halted. This switch-like change in cholesterol accessibility is observed not only in purified ER membranes but also in liposomes made from ER lipid extracts. The accessibility of cholesterol in membranes is related to its chemical activity. Complex formation between cholesterol and some ER phospholipids can result in sharp changes in cholesterol chemical activity and its accessibility to perfringolysin O or membrane sensors like Scap. The control of the availability of the cholesterol ligand to participate in cooperative Scap/cholesterol/Insig interactions further sharpens the sensitive switch that exerts precise control over cholesterol levels in cell membranes.

3.1516 Sub-proteome approach to the knowledge of liver
Falcon-Perez, J.M., Lu, S.C. and Mato, J.M.
Proteomics Clin. Appl., ***4***, *407-415 (2010)*

In the recent years, global proteomics approaches have been widely used to characterize a number of tissue proteomes including plasma and liver; however, the elevated complexity of these samples in combination with the high abundance of some specific proteins make the study of the lowest abundant proteins difficult. This review is focused on different strategies that have been developed to extend the proteome focused on these two tissues, as, for example, the analysis of sub-cellular proteomes. In this regard, two special kind of extracellular vesicles – exosomes and membrane plasma shedding vesicles – are emerging as excellent biological source both to extend the liver and plasma proteomes and to be applied in the discovery of non-invasive liver-specific disease biomarkers.

3.1517 Urokinase Plasminogen Activator Receptor and/or Matrix Metalloproteinase-9 Inhibition Induces Apoptosis Signaling through Lipid Rafts in Glioblastoma Xenograft Cells
Chetty, C., Lakka, S.S., Bhoopathi, P., Gondi, C.S., Veeravalli, K.K., Fassett, D., Klopfenstein, J.D., Dinh, D.H., Gujrati, M. and Rao, J.S.
Mol. Cancer Ther., ***9***, *2605-2617 (2010)*

Small interfering RNA (siRNA)-mediated transcriptional knockdown of urokinase plasminogen activator receptor (uPAR) and matrix metalloproteinase-9 (MMP-9), alone or in combination, inhibits uPAR and/or MMP-9 expression and induces apoptosis in the human glioblastoma xenograft cell lines 4910 and 5310. siRNA against uPAR (pU-Si), MMP-9 (pM-Si), or both (pUM-Si) induced apoptosis and was associated with the cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase. Furthermore, protein levels of the Fas receptor (APO-1/CD-95) were increased following transcriptional inactivation of uPAR and/or MMP-9. In addition, Fas siRNA against the Fas death receptor blocked apoptosis induced by pU-Si, pM-Si, or pUM-Si, thereby indicating the role for Fas signaling in pU-Si–, pM-Si–, or pUM-Si–mediated

apoptotic cell death of human glioma xenograft cells. Thus, transcriptional inactivation of uPAR and/or MMP-9 enhanced localization of Fas death receptor, Fas-associated death domain-containing protein, and procaspase-8 into lipid rafts. Additionally, disruption of lipid rafts with methyl β cyclodextrin prevented Fas clustering and pU-Si–, pM-Si–, or pUM-Si–induced apoptosis, which is indicative of coclustering of Fas death receptor into lipid rafts in the glioblastoma xenograft cell lines 4910 and 5310. These data indicate the crucial role of the clusters of apoptotic signaling molecule-enriched rafts in programmed cell death, acting as concentrators of death receptors and downstream signaling molecules, and as the linchpin from which a potent death signal is launched in uPAR- and/or MMP-9–downregulated cells.

3.1518 m-Calpain-mediated cleavage of Na^+/Ca^{2+} exchanger-1 in caveolae vesicles isolated from pulmonary artery smooth muscle

Shaikh, S., Samanta, K., Kar, P., Roy, S., Chakraborti, T. and Chakraborti, S.
Mol. Cell. Biochem., ***341***, *167-180 (2010)*

Using m-calpain antibody, we have identified two major bands corresponding to the 80 kDa large and the 28 kDa small subunit of m-calpain in caveolae vesicles isolated from bovine pulmonary artery smooth muscle plasma membrane. In addition, 78, 35, and 18 kDa immunoreactive bands of m-calpain have also been detected. Casein zymogram studies also revealed the presence of m-calpain in the caveolae vesicles. We have also identified Na^+/Ca^{2+} exchanger-1 (NCX1) in the caveolae vesicles. Purification and N-terminal sequence analyses of these two proteins confirmed their identities as m-calpain and NCX1, respectively. We further sought to determine the role of m-calpain on calcium-dependent proteolytic cleavage of NCX1 in the caveolae vesicles. Treatment of the caveolae vesicles with the calcium ionophore, A23187 (1 μM) in presence of $CaCl_2$ (1 mM) appears to cleave NCX1 (120 kDa) to an 82 kDa fragment as revealed by immunoblot study using NCX1 monoclonal antibody; while pretreatment with the calpain inhibitors, calpeptin or MDL28170; or the Ca^{2+} chelator, BAPTA-AM did not cause a discernible change in the NCX protein profile. In vitro cleavage of the purified NCX1 by the purified m-calpain supports this finding. The cleavage of NCX1 by m-calpain in the caveolae vesicles may be interpreted as an important mechanism of Ca^{2+} overload, which could arise due to inhibition of Ca^{2+} efflux by the forward-mode NCX and that could lead to sustained Ca^{2+} overload in the smooth muscle leading to pulmonary hypertension.

3.1519 The Intracellular Localization and Function of the ATP-Sensitive K+ Channel Subunit Kir6.1

Ng, K-E., Schwarzer, S., Duchen, M.R. and Tinker, A.
J. Membrane Biol., ***234***, *137-147 (2010)*

Our aim was to determine the subcellular localization and functional roles of the K_{ATP} channel subunit Kir6.1 in intracellular membranes. Specifically, we focused on the potential role of Kir6.1 as a subunit of the mitochondrial ATP-sensitive K^+ channel. Cell imaging showed that a major proportion of heterologously expressed Kir6.1-GFP and endogenously expressed Kir6.1 was distributed in the endoplasmic reticulum with little in the mitochondria or plasma membrane. We used pharmacological and molecular tools to investigate the functional significance of this distribution. The K_{ATP} channel opener diazoxide increased reactive oxygen species production, and glibenclamide abolished this effect. However, in cells lacking Kir6.1 or expressing siRNA or dominant negative constructs of Kir6.1, the same effect was seen. Ca^{2+} handling was examined in the muscle cell line C2C12. Transfection of the dominant negative constructs of Kir6.1 significantly reduced the amplitude and rate of rise of $[Ca^{2+}]_c$ transients elicited by ATP. This study suggests that Kir6.1 is located in the endoplasmic reticulum and plays a role in modifying Ca^{2+} release from intracellular stores.

3.1520 Ero1α requires oxidizing and normoxic conditions to localize to the mitochondria-associated membrane (MAM)

Gilady, S., Bui, M., Lynes, E.M., Benson, M.D., Watts, R., Vance, J.E. and Simmen, T.
Cell Stress and Chaperones, ***15***, *619-629 (2010)*

Protein secretion from the endoplasmic reticulum (ER) requires the enzymatic activity of chaperones and oxidoreductases that fold polypeptides and form disulfide bonds within newly synthesized proteins. The best-characterized ER redox relay depends on the transfer of oxidizing equivalents from molecular oxygen through ER oxidoreductin 1 (Ero1) and protein disulfide isomerase to nascent polypeptides. The formation of disulfide bonds is, however, not the sole function of ER oxidoreductases, which are also important regulators of ER calcium homeostasis. Given the role of human Ero1α in the regulation of the calcium release by inositol 1,4,5-trisphosphate receptors during the onset of apoptosis, we hypothesized that Ero1α may have a redox-sensitive localization to specific domains of the ER. Our results show that within the ER,

Ero1α is almost exclusively found on the mitochondria-associated membrane (MAM). The localization of Ero1α on the MAM is dependent on oxidizing conditions within the ER. Chemical reduction of the ER environment, but not ER stress in general leads to release of Ero1α from the MAM. In addition, the correct localization of Ero1α to the MAM also requires normoxic conditions, but not ongoing oxidative phosphorylation.

3.1521 Compartmentalization of EGFR in cellular membranes: Role of membrane rafts
Balbis, A. and Posner, B.I.
J. Cell. Biochem., ***109****, 1103-1108 (2010)*

There is now abundant evidence that the intracellular concentration of the EGFR and many other receptors for peptide hormones and growth factors is important for the temporal and spatial regulation of cell signaling. Spatial control is achieved by the selective compartmentalization of signaling components into endosomes. However further control may be effected by sequestration into sub-domains within a given organelle such as membrane rafts which are dynamic, nano scale structures rich in cholesterol and sphingolipids. Current data suggest the presence of EGFRs in non-caveolae membrane rafts. High doses of EGF seem to promote the sorting of EGFR to late endosomes through a raft/cholesterol dependant mechanism, implicating them in EGFR degradation. However our work and that of others has led us to propose a model in which membrane rafts in late endosomes sequester highly active EGFR leading to the recruitment and activation of MAPK in this compartment.

3.1522 Long chain-polyunsaturated fatty acids modulate membrane phospholipid composition and protein localization in lipid rafts of neural stem cell cultures
Langelier, B., Linard, A., Bordat, C., Lavialle, M. and Heberden, C.
J. Cell. Biochem., ***110****, 1356-1364 (2010)*

Rat neural stem cells/neural progenitors (NSC/NP) are generally grown in serum-free medium. In this study, NSC/NP were supplemented with the main long-chain polyunsaturated fatty acids (PUFAs) present in the brain, arachidonic acid (AA), or docosahexaenoic acid (DHA), and were monitored for their growth. Lipid and fatty acid contents of the cells were also determined. Under standard conditions, the cells were characterized by phospholipids displaying a highly saturated profile, and very low levels of PUFAs. When cultured in the presence of PUFAs, the cells easily incorporated them into the phospholipid fraction. We also compared the presence of three membrane proteins in the lipid raft fractions: GFR and connexin 43 contents in the rafts were increased by DHA supplementation, whereas Gβ subunit content was not significantly modified. The restoration of DHA levels in the phospholipids could profoundly affect protein localization and, consequently, their functionalities.

3.1523 Sphingomyelin is important for the cellular entry and intracellular localization of Helicobacter pylori VacA
Gupta, V.R., Wilson, B.A. and Blanke, S.R.
Cellular Microbiol., ***12(10)****, 1517-1533 (2010)*

Plasma membrane sphingomyelin (SM) binds the *Helicobacter pylori* vacuolating toxin (VacA) to the surface of epithelial cells. To evaluate the importance of SM for VacA cellular entry, we characterized toxin uptake and trafficking within cells enriched with synthetic variants of SM, whose intracellular trafficking properties are strictly dependent on the acyl chain lengths of their sphingolipid backbones. While toxin binding to the surface of cells was independent of acyl chain length, cells enriched with 12- or 18-carbon acyl chain variants of SM (e.g. C12-SM or C18-SM) were more sensitive to VacA, as indicated by toxin-induced cellular vacuolation, than those enriched with shorter 2- or 6-carbon variants (e.g. C2-SM or C6-SM). In C18-SM-enriched cells, VacA was taken into cells by a previously described Cdc42-dependent pinocytic mechanism, localized initially to GPI-enriched vesicles, and ultimately trafficked to Rab7/Lamp1 compartments. In contrast, within C2-SM-enriched cells, VacA was taken up at a slower rate by a Cdc42-independent mechanism and trafficked to Rab11 compartments. VacA-associated predominantly with detergent-resistant membranes (DRMs) in cells enriched with C18-SM, but predominantly with non-DRMs in C2-SM-enriched cells. These results suggest that SM is required for targeting VacA to membrane rafts important for subsequent Cdc42-dependent pinocytic cellular entry.

3.1524 Ttyh1, a Ca^{2+}-binding protein localized to the endoplasmic reticulum, is required for early embryonic development

Kumada, T., Yamanaka, Y., Kitano, A., Shibata, M., Awaya, T., Kato, T., Okawa, K., Abe, T., Oshima, N., Nakahata, T. and Heike, T.
*Developmental Dynamics, **239**, 2233-2245 (2010)*

Using comprehensive genetic studies on neuronal stem/progenitors cells through genome-wide screening with oligonucleotide arrays, we identified an endoplasmic reticulum (ER) -resident protein, *Tweety homologue 1* (*ttyh1*). *Ttyh1* encodes a glycosylated protein composed of five predicted transmembrane segments and a C-terminus that is enriched in negatively charged residues capable of Ca^{2+} binding. Ttyh1-containing membranes changed to segmented tubuloreticular structures during mitosis, suggesting that the ER-containing Ttyh1 could be responsible for Ca^{2+} sequestration and Ca^{2+} concentration regulation during mitosis. *Ttyh1* inactivation in mice resulted in early embryonic lethality before organization of the nervous system, revealing that *ttyh1* is essential in murine embryonic development. Our findings indicate that Ttyh1 plays an indispensable role during mitosis in early embryogenesis, possibly by maintaining Ca^{2+} homeostasis in the ER.

3.1525 A proteomic approach towards the identification of the matrix protein content of the two types of microbodies in *Neurospora crassa*

Managadze, D., Würtz, C., Wiese, S., Meyer, H.E., Niehaus, G., Erdmann, R., Warscheid, B and Rottensteiner, H.
*Proteomics, **10**, 3222-3234 (2010)*

Microbodies (peroxisomes) comprise a class of organelles with a similar biogenesis but remarkable biochemical heterogeneity. Here, we purified the two distinct microbody family members of filamentous fungi, glyoxysomes and Woronin bodies, from *Neurospora crassa* and analyzed their protein content by HPLC/ESI-MS/MS. In the purified Woronin bodies, we unambiguously identified only hexagonal 1 (HEX1), suggesting that the matrix is probably exclusively filled with the HEX1 hexagonal crystal. The proteomic analysis of highly purified glyoxysomes allowed the identification of 191 proteins. Among them were 16 proteins with a peroxisomal targeting signal type 1 (PTS1) and three with a PTS2. The collection also contained the previously described *N. crassa* glyoxysomal matrix proteins FOX2 and ICL1 that lack a typical PTS. Three PTS1 proteins were identified that likely represent the long sought glyoxysomal acyl-CoA dehydrogenases of filamentous fungi. Two of them were demonstrated by subcellular localization studies to be indeed glyoxysomal. Furthermore, two PTS proteins were identified that are suggested to be involved in the detoxification of nitroalkanes. Since the glyoxysomal localization was experimentally demonstrated for one of these enzymes, a new biochemical reaction is expected to be associated with microbody function.

3.1526 PLP/DM20 Expression and turnover in a transgenic mouse model of pelizaeus-merzbacher disease

Karim, S.A., Barrie, J., McCulloch, M.C., Montaque, P., Edgar, J.M., Iden, D.L., Anderson, T.J., Nave, K-A., Griffiths, I.R. and McLaughlin, M.
*Glia, **58**, 1727-1738 (2010)*

The most common cause of Pelizaeus-Merzbacher (PMD) is due to duplication of the *PLP1* gene but it is unclear how increased gene dosage affects PLP turnover and causes dysmyelination. We have studied the dynamics of PLP/DM20 in a transgenic mouse model of PMD with increased gene dosage of the proteolipid protein gene (*Plp1*). The turnover of PLP/DM20 were investigated using an *ex-vivo* brain slice system and cultured oligodendrocytes. Homozygous mice have reduced PLP translation, markedly enhanced PLP degradation, and markedly reduced incorporation of PLP into myelin. Proteasome inhibition (MG132) prevented the enhanced degradation. Numerous autophagic vesicles are present in homozygous transgenic mice that may influence protein dynamics. Surprisingly, promoting autophagy with rapamycin decreases the degradation of nascent PLP suggesting autophagic vacuoles serve as a cellular storage compartment. We suggest that there are multiple subcellular fates of PLP/DM20 when overexpressed: the vast majority being degraded by the proteasome, a proportion sequestered into autophagic vacuoles, probably fused with endolysosomes, and only a small proportion entering the myelin sheath, where its association with lipid rafts is perturbed. Transgenic oligodendrocytes have fewer membrane sheets and this phenotype is improved with siRNA-mediated knockdown of PLP expression that promotes the formation of MBP+ myelin-like sheets. This finding suggests that RNAi technology is in principle applicable to improve CNS myelination when compromised by PLP/DM20 overexpression.

3.1527 **The Versatility of Helicobacter pylori CagA Effector Protein Functions: The Master Key Hypothesis**
Backert, S., Tegtmeyer, N. and Selbach, M.
Helicobacter, ***15****, 163-176 (2010)*

Several bacterial pathogens inject virulence proteins into host target cells that are substrates of eukaryotic tyrosine kinases. One of the key examples is the *Helicobacter pylori* CagA effector protein which is translocated by a type-IV secretion system. Injected CagA becomes tyrosine-phosphorylated on EPIYA sequence motifs by Src and Abl family kinases. CagA then binds to and activates/inactivates multiple signaling proteins in a phosphorylation-dependent and phosphorylation-independent manner. A recent proteomic screen systematically identified eukaryotic binding partners of the EPIYA phosphorylation sites of CagA and similar sites in other bacterial effectors by high-resolution mass spectrometry. Individual phosphorylation sites recruited a surprisingly high number of interaction partners suggesting that each phosphorylation site can interfere with many downstream pathways. We now count 20 reported cellular binding partners of CagA, which represents the highest quantitiy among all yet known virulence-associated effector proteins in the microbial world. This complexity generates a highly remarkable and puzzling scenario. In addition, the first crystal structure of CagA provided us with new information on the function of this important virulence determinant. Here we review the recent advances in characterizing the multiple binding signaling activities of CagA. Injected CagA can act as a 'master key' that evolved the ability to highjack multiple host cell signalling cascades, which include the induction of membrane dynamics, actin-cytoskeletal rearrangements and the disruption of cell-to-cell junctions as well as proliferative, pro-inflammatory and anti-apoptotic nuclear responses. The discovery that different pathogens use this common strategy to subvert host cell functions suggests that more examples will emerge soon.

3.1528 **Disruption of cellular cholesterol transport and homeostasis as a novel mechanism of action of membrane-targeted alkylphospholipid analogues**
Carrasco, M:P., Jimenez-Lopez, J.M., Rios-Marco, P., Segovia, J.L. and Marco, C.
Br. J. Pharmacol., ***160****, 355-366 (2010)*

Background and purpose: Alkylphospholipid (APL) analogues constitute a new class of synthetic anti-tumour agents that act directly on cell membranes. We have previously demonstrated that hexadecylphosphocholine (HePC) alters intracellular cholesterol traffic and metabolism in HepG2 cells. We now extended our studies to analyse the effects of other clinically relevant APLs, such as edelfosine, erucylphosphocholine and perifosine on intracellular cholesterol homeostasis.
Experimental approach: Using radiolabelled substrates we determined the effect of APLs on cholesterol metabolism and cholesterol traffic from the plasma membrane to the endoplasmic reticulum (ER). Protein levels and gene expression of the main proteins involved in cholesterol homeostasis were analysed by Western blot and RT-PCR respectively. Membrane raft and non-raft fractions were isolated from HepG2 cells by a detergent-free method.
Key results: All APLs inhibited the transport of cholesterol from the plasma membrane to the ER, which induced a significant cholesterogenic response in HepG2 cells. This response involved an increased gene expression and higher levels of several proteins related to the biosynthesis and the receptor-mediated uptake of cholesterol. Cell exposure to the APL-representative HePC enhanced the content of cholesterol mainly in the membrane raft fractions, compared with the untreated cells.
Conclusions and implications: Membrane-targeted APLs exhibited a novel and common mechanism of action, through disruption of cholesterol homeostasis, which in turn affected specific lipid microdomains of cellular membranes.

3.1529 **The Arabidopsis Peroxisomal ABC Transporter, Comatose, Complements the Saccharomyces cerevisiae pxa1 pxa2Δ Mutant for Metabolism of Long-chain Fatty Acids and Exhibits Fatty Acyl-CoA-stimulated ATPase Activity**
Nyathi, Y., De Marcos Lousa, C., van Roermund, C.W., Wanders, R.J.A., Johnson, B., Baldwin, S.A., Theodoulou, F.L. and Baker, A.
J. Biol. Chem., ***285(38)****, 29892-29902 (2010)*

The *Arabidopsis* ABC transporter Comatose (CTS; AtABCD1) is required for uptake into the peroxisome of a wide range of substrates for β-oxidation, but it is uncertain whether CTS itself is the transporter or if the transported substrates are free acids or CoA esters. To establish a system for its biochemical analysis, CTS was expressed in *Saccharomyces cerevisiae*. The plant protein was correctly targeted to yeast peroxisomes, was assembled into the membrane with its nucleotide binding domains in the cytosol, and exhibited basal ATPase activity that was sensitive to aluminum fluoride and abrogated by mutation of a

conserved Walker A motif lysine residue. The yeast *pxa1 pxa2*Δ mutant lacks the homologous peroxisomal ABC transporter and is unable to grow on oleic acid. Consistent with its exhibiting a function in yeast akin to that in the plant, CTS rescued the oleate growth phenotype of the *pxa1 pxa2*Δ mutant, and restored β-oxidation of fatty acids with a range of chain lengths and varying degrees of desaturation. When expressed in yeast peroxisomal membranes, the basal ATPase activity of CTS could be stimulated by fatty acyl-CoAs but not by fatty acids. The implications of these findings for the function and substrate specificity of CTS are discussed.

3.1530 Agonist-Specific Compartmentation of cGMP Action in Myometrium
Buxton, I.L.O., Milton, D., Barnett, S.D. and Tichenor, S.D.
J. Pharmacol. Exp. Ther., ***335(1)***, *256-263 (2010)*

Nitric oxide relaxes myometrium in a cGMP-independent manner. Although cGMP activates its cognate kinase, this is not required for the inhibitory effect of nitric oxide. Thus, nitric oxide-mediated cGMP elevation does not enjoy the same set of substrates as it does in other smooth muscles. To further understand the regulation of relaxation of uterine muscle by cGMP, we have studied the actions of peptide-mediated cGMP action in guinea pig myometrium. We used both functional and biochemical studies of the action of the particulate guanylyl cyclase activator uroguanylin and its receptor, particulate guanylyl cyclase type C, to address the relationship between cGMP elevation acting in the membrane signaling domain to that of the nonmembrane region of the cell. Uroguanylin relaxed oxytocin-induced contractions in a dose-dependent fashion only in pregnant myometrium. Both relaxation and cGMP accumulation after uroguanylin stimulation were blocked by the putative particulate guanylyl cyclase type C inhibitors 2-chloro-ATP and isatin (1*H*-indole-2,3-dione), but not by the soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-A]quinoxalin-1-one (ODQ). Uroguanylin stimulated cGMP accumulation only in the pregnant myometrium. Caveolin-1 expression increased in pregnancy toward term. In the caveolin-1-containing membrane domain, uroguanylin, but not the nitric-oxide donor, led to the elevation of cGMP that was insensitive to ODQ. Particulate guanylyl cyclase C was expressed and prouroguanylin was detected in pregnant myometrium. We conclude that a uroguanylin–particulate cyclase-cGMP relaxation pathway is present and cGMP is compartmented in myometrium. The agonist-mediated selectivity of relaxation to cGMP is of fundamental pharmacological interest in understanding signal transduction in smooth muscle.

3.1531 Inorganic polyphosphate inhibits an aspartic protease-like activity in the eggs of Rhodnius prolixus (Stahl) and impairs yolk mobilization in vitro
Gomes, F.M., Oliveira, D.M.P., Motta, L.S., Ramos, I.B., Miranda, K.M. and Macchado, E.A.
J. Cell. Physiol., ***222***, *606-611 (2010)*

Inorganic polyphosphate (poly P) is a polymer of phosphate residues that has been shown to act as modulator of some vertebrate cathepsins. In the egg yolk granules of *Rhodnius prolixus*, a cathepsin D is the main protease involved in yolk mobilization and is dependent on an activation by acid phosphatases. In this study, we showed a possible role of poly P stored inside yolk granules on the inhibition of cathepsin D and arrest of yolk mobilization during early embryogenesis of these insects. Enzymatic assays detected poly P stores inside the eggs of *R. prolixus*. We observed that micromolar poly P concentrations inhibited cathepsin D proteolytic activity using both synthetic peptides and homogenates of egg yolk as substrates. Poly P was a substrate for *Rhodnius* acid phosphatase and also a strong competitive inhibitor of a pNPPase activity. Fusion events have been suggested as important steps towards acid phosphatase transport to yolk granules. We observed that poly P levels in those compartments were reduced after in vitro fusion assays and that the remaining poly P did not have the same cathepsin D inhibition activity after fusion. Our results are consistent with the hypothesis that poly P is a cathepsin D inhibitor and a substrate for acid phosphatase inside yolk granules. It is possible that, once activated, acid phosphatase might degrade poly P, allowing cathepsin D to initiate yolk proteolysis. We, therefore, suggest that degradation of poly P might represent a new step toward yolk mobilization during embryogenesis of *R. prolixus.*

3.1532 Acylation-dependent Export of Trypanosoma cruzi Phosphoinositide-specific Phospholipase C to the Outer Surface of Amastigotes
De Paulo Martins, V., Okura, M., Maric, D., Engman, D.M., Vieira, M., Docompo, R. and Moreno, S.N.J.
J. Biol. Chem., ***285(40)***, *30906-30917 (2010)*

Phosphoinositide phospholipase C (PI-PLC) plays an essential role in cell signaling. A unique *Trypanosoma cruzi* PI-PLC (*Tc*PI-PLC) is lipid-modified in its N terminus and localizes to the plasma membrane of amastigotes. Here, we show that *Tc*PI-PLC is located onto the extracellular phase of the plasma membrane of amastigotes and that its N-terminal 20 amino acids are necessary and sufficient to target the fused GFP to the outer surface of the parasite. Mutagenesis of the predicted acylated residues confirmed that myristoylation of a glycine residue in the 2nd position and acyl modification of a cysteine in the 4th but not in the 8th or 15th position of the coding sequence are required for correct plasma membrane localization in *T. cruzi* epimastigotes or amastigotes. Interestingly, mutagenesis of the cysteine at the 8th position increased its flagellar localization. When expressed as fusion constructs with GFP, the N-terminal 6 and 10 amino acids fused to GFP are predominantly located in the cytosol and concentrated in a compartment that co-localizes with a Golgi complex marker. The N-terminal 20 amino acids of *Tc*PI-PLC associate with lipid rafts when dually acylated. Taken together, these results indicate that N-terminal acyl modifications serve as a molecular addressing system for sending *Tc*PI-PLC to the outer surface of the cell.

3.1533 Rab32 Modulates Apoptosis Onset and Mitochondria-associated Membrane (MAM) Properties
Bui, M., Gilady, S.Y., Fitzsimmons, R.E.B., Benson, M.D., Lynes, E.M., Gesson, K., Alto, N.M., Strack, S., Scott, J.D. and Simmen, T.
J. Biol. Chem., ***285(41)***, *31590-31602 (2010)*

The mitochondria-associated membrane (MAM) has emerged as an endoplasmic reticulum (ER) signaling hub that accommodates ER chaperones, including the lectin calnexin. At the MAM, these chaperones control ER homeostasis but also play a role in the onset of ER stress-mediated apoptosis, likely through the modulation of ER calcium signaling. These opposing roles of MAM-localized chaperones suggest the existence of mechanisms that regulate the composition and the properties of ER membrane domains. Our results now show that the GTPase Rab32 localizes to the ER and mitochondria, and we identify this protein as a regulator of MAM properties. Consistent with such a role, Rab32 modulates ER calcium handling and disrupts the specific enrichment of calnexin on the MAM, while not affecting the ER distribution of protein-disulfide isomerase and mitofusin-2. Furthermore, Rab32 determines the targeting of PKA to mitochondrial and ER membranes and through its overexpression or inactivation increases the phosphorylation of Bad and of Drp1. Through a combination of its functions as a PKA-anchoring protein and a regulator of MAM properties, the activity and expression level of Rab32 determine the speed of apoptosis onset.

3.1534 Multivesicular Body Formation Requires OSBP-Related Proteins and Cholesterol
Kobuna, H., Inoue, T., Shibata, M., Gengyo-Ando, K., Yamamoto, A., Mitani, S. and Arai, H.
PloS Genetics, ***6(8)***, *e1001055 (2010)*

In eukaryotes, different subcellular organelles have distinct cholesterol concentrations, which is thought to be critical for biological functions. Oxysterol-binding protein-related proteins (ORPs) have been assumed to mediate nonvesicular cholesterol trafficking in cells; however, their *in vivo* functions and therefore the biological significance of cholesterol in each organelle are not fully understood. Here, by generating deletion mutants of ORPs in *Caenorhabditis elegans*, we show that ORPs are required for the formation and function of multivesicular bodies (MVBs). In an RNAi enhancer screen using *obr* quadruple mutants (*obr-1; -2; -3; -4*), we found that MVB–related genes show strong genetic interactions with the *obr* genes. In *obr* quadruple mutants, late endosomes/lysosomes are enlarged and membrane protein degradation is retarded, although endocytosed soluble proteins are normally delivered to lysosomes and degraded. We also found that the cholesterol content of late endosomes/lysosomes is reduced in the mutants. In wild-type worms, cholesterol restriction induces the formation of enlarged late endosomes/lysosomes, as observed in *obr* quadruple mutants, and increases embryonic lethality upon knockdown of MVB–related genes. Finally, we show that knockdown of ORP1L, a mammalian ORP family member, induces the formation of enlarged MVBs in HeLa cells. Our *in vivo* findings suggest that the proper cholesterol level of late endosomes/lysosomes generated by ORPs is required for normal MVB formation and MVB–mediated membrane protein degradation.

3.1535 A Novel Role for the Centrosomal Protein, Pericentrin, in Regulation of Insulin Secretory Vesicle Docking in Mouse Pancreatic β-cells
Jurczyk, A., Pino, S.C., O'Sullivan-Murphy, B., Addorio, M., Lidstone, E.A., Dilorio, P., Lipson, K.-L., Standley, C., Fogarty, K., Lifshitz, L., Urano, F., Mordes, J.P., Greiner, D.L., Rossini, A.A. and Bortell, R.
PloSOne, ***5(7)***, *e11812 (2010)*

The centrosome is important for microtubule organization and cell cycle progression in animal cells. Recently, mutations in the centrosomal protein, pericentrin, have been linked to human microcephalic osteodysplastic primordial dwarfism (MOPD II), a rare genetic disease characterized by severe growth retardation and early onset of type 2 diabetes among other clinical manifestations. While the link between centrosomal and cell cycle defects may account for growth deficiencies, the mechanism linking pericentrin mutations with dysregulated glucose homeostasis and pre-pubertal onset of diabetes is unknown. In this report we observed abundant expression of pericentrin in quiescent pancreatic β-cells of normal animals which led us to hypothesize that pericentrin may have a critical function in β-cells distinct from its known role in regulating cell cycle progression. In addition to the typical centrosome localization, pericentrin was also enriched with secretory vesicles in the cytoplasm. Pericentrin overexpression in β-cells resulted in aggregation of insulin-containing secretory vesicles with cytoplasmic, but not centrosomal, pericentriolar material and an increase in total levels of intracellular insulin. RNAi- mediated silencing of pericentrin in secretory β-cells caused dysregulated secretory vesicle hypersecretion of insulin into the media. Together, these data suggest that pericentrin may regulate the intracellular distribution and secretion of insulin. Mice transplanted with pericentrin-depleted islets exhibited abnormal fasting hypoglycemia and inability to regulate blood glucose normally during a glucose challenge, which is consistent with our in vitro data. This previously unrecognized function for a centrosomal protein to mediate vesicle docking in secretory endocrine cells emphasizes the adaptability of these scaffolding proteins to regulate diverse cellular processes and identifies a novel target for modulating regulated protein secretion in disorders such as diabetes.

3.1536 Posttranslational mechanisms associated with reduced NHE3 activity in adult vs. young prehypertensive SHR

Crajoinas, R.O., Lessa, L.M.A., Carraro-Lacroix, L.R., Davel, A.P.C., pachero, B.P.M., Rossoni, L.V., Malnic, G. and Girardi, A.C.C.

Am. J. Physiol. Renal Physiol., ***299****, F872-F881 (2010)*

Abnormalities in renal proximal tubular (PT) sodium transport play an important role in the pathophysiology of essential hypertension. The Na^+/H^+ exchanger isoform 3 (NHE3) represents the major route for sodium entry across the apical membrane of renal PT cells. We therefore aimed to assess in vivo NHE3 transport activity and to define the molecular mechanisms underlying NHE3 regulation before and after development of hypertension in the spontaneously hypertensive rat (SHR). NHE3 function was measured as the rate of bicarbonate reabsorption by means of in vivo stationary microperfusion in PT from young prehypertensive SHR (Y-SHR; 5-wk-old), adult SHR (A-SHR; 14-wk-old), and age-matched Wistar Kyoto (WKY) rats. We found that NHE3-mediated PT bicarbonate reabsorption was reduced with age in the SHR (1.08 ± 0.10 vs. 0.41 ± 0.04 nmol/cm^{2}xs), while it was increased in the transition from youth to adulthood in the WKY rat (0.59 ± 0.05 vs. 1.26 ± 0.11 nmol/cm^{2}xs). Higher NHE3 activity in the Y-SHR compared with A-SHR was associated with a predominant microvilli confinement and a lower ratio of phosphorylated NHE3 at serine-552 to total NHE3 (P-NHE3/total). After development of hypertension, P-NHE3/total increased and NHE3 was retracted out of the microvillar microdomain along with the regulator dipeptidyl peptidase IV (DPPIV). Collectively, our data suggest that the PT is playing a role in adapting to the hypertension in the SHR. The molecular mechanisms of this adaptation possibly include an increase of P-NHE3/total and a redistribution of the NHE3-DPPIV complex from the body to the base of the PT microvilli, both predicted to decrease sodium reabsorption.

3.1537 Unexpected Role of the Copper Transporter ATP7A in PDGF-Induced Vascular Smooth Muscle Cell Migration

Ashino, T., Sudhahar, V., Yrao, N., Oshikawa, J., Chen, G-F., Wang, H., Huo, Y., Finney, L., Vogt, S., McKinney, R.D., Maryon, E.B., Kaplan, J.H., Ushio-Fakai, M. and Fukai, T.

Circ. Res., ***107****, 787-799 (2010)*

Rationale:Copper, an essential nutrient, has been implicated in vascular remodeling and atherosclerosis with unknown mechanism. Bioavailability of intracellular copper is regulated not only by the copper importer CTR1 (copper transporter 1) but also by the copper exporter ATP7A (Menkes ATPase), whose function is achieved through copper-dependent translocation from *trans*-Golgi network (TGN). Platelet-derived growth factor (PDGF) promotes vascular smooth muscle cell (VSMC) migration, a key component of neointimal formation.

Objective:To determine the role of copper transporter ATP7A in PDGF-induced VSMC migration.

Methods and Results:Depletion of ATP7A inhibited VSMC migration in response to PDGF or wound scratch in a CTR1/copper-dependent manner. PDGF stimulation promoted ATP7A translocation from the TGN to lipid rafts, which localized at the leading edge, where it colocalized with PDGF receptor and Rac1, in migrating VSMCs. Mechanistically, ATP7A small interfering RNA or CTR small interfering RNA prevented PDGF-induced Rac1 translocation to the leading edge, thereby inhibiting lamellipodia formation. In addition, ATP7A depletion prevented a PDGF-induced decrease in copper level and secretory copper enzyme precursor prolysyl oxidase (Pro-LOX) in lipid raft fraction, as well as PDGF-induced increase in LOX activity. In vivo, ATP7A expression was markedly increased and copper accumulation was observed by synchrotron-based x-ray fluorescence microscopy at neointimal VSMCs in wire injury model. ***Conclusions:***These findings suggest that ATP7A plays an important role in copper-dependent PDGF-stimulated VSMC migration via recruiting Rac1 to lipid rafts at the leading edge, as well as regulating LOX activity. This may contribute to neointimal formation after vascular injury. Our findings provide insight into ATP7A as a novel therapeutic target for vascular remodeling and atherosclerosis.

3.1538 Role of EBAG9 protein in coat protein complex I-dependent glycoprotein maturation and secretion processes in tumor cells

Wolf, J., Reimer, T.A., Schuck, S., Rüder, C., Gerlach, K., Müller, E-C., Otto, A., Dörken, B. and Rehm, A.

*FASEB J., **24**, 4000-4019 (2010)*

Many proteins mature within the secretory pathway by the acquisition of glycans. Failure to maintain the proper distribution of the glycosylation machinery might lead to disease. High expression levels of the ubiquitous Golgi protein estrogen receptor-binding fragment-associated gene 9 (EBAG9) in human tumors correlate with poor clinical prognosis, and EBAG9 overexpression in epithelial cell lines induces truncated glycans, typical of many carcinomas. Here, we addressed the pathogenetic link between EBAG9 expression and the alteration of the cellular glycome. We applied confocal microscopy, live imaging, pulse-chase labeling in conjunction with immunoprecipitation, and enzymatic activity assays in a variety of EBAG9-overexpressing or depleted epithelial tumor cell lines. EBAG9 shuttles between the ER-Golgi intermediate compartment and the *cis*-Golgi, and we demonstrate association of EBAG9 with coat protein complex I (COPI)-coated transport vesicles. EBAG9 overexpression imposes delay of endoplasmic reticulum-to-Golgi transport and mislocalizes components of the ER quality control and glycosylation machinery. Conversely, EBAG9 down-regulation accelerates glycoprotein transport through the Golgi and enhances mannosidase activity. Thus, EBAG9 acts as a negative regulator of a COPI-dependent ER-to-Golgi transport pathway in epithelial cells and represents a novel pathogenetic principle in which interference with intracellular membrane trafficking results in the emergence of a tumor-associated glycome.

3.1539 Pathogenic Lysosomal Depletion in Parkinson's Disease

Dehay, B., Bove, J., Rodriguez-Muela, N., Perier, C., Recases, A., Boya, P. and Vila, M.

*J. Neurosci., **30(37)**, 12535-12544 (2010)*

Mounting evidence suggests a role for autophagy dysregulation in Parkinson's disease (PD). The bulk degradation of cytoplasmic proteins (including α-synuclein) and organelles (such as mitochondria) is mediated by macroautophagy, which involves the sequestration of cytosolic components into autophagosomes (AP) and its delivery to lysosomes. Accumulation of AP occurs in postmortem brain samples from PD patients, which has been widely attributed to an induction of autophagy. However, the cause and pathogenic significance of these changes remain unknown. Here we found in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD that AP accumulation and dopaminergic cell death are preceded by a marked decrease in the amount of lysosomes within dopaminergic neurons. Lysosomal depletion was secondary to the abnormal permeabilization of lysosomal membranes induced by increased mitochondrial-derived reactive oxygen species. Lysosomal permeabilization resulted in a defective clearance and subsequent accumulation of undegraded AP and contributed directly to neurodegeneration by the ectopic release of lysosomal proteases into the cytosol. Lysosomal breakdown and AP accumulation also occurred in PD brain samples, where Lewy bodies were strongly immunoreactive for AP markers. Induction of lysosomal biogenesis by genetic or pharmacological activation of lysosomal transcription factor EB restored lysosomal levels, increased AP clearance and attenuated 1-methyl-4-phenylpyridinium-induced cell death. Similarly, the autophagy-enhancer compound rapamycin attenuated PD-related dopaminergic neurodegeneration, both *in vitro* and *in vivo*, by restoring lysosomal levels. Our results indicate that AP accumulation in PD results from defective lysosomal-mediated AP clearance secondary to

lysosomal depletion. Restoration of lysosomal levels and function may thus represent a novel neuroprotective strategy in PD.

3.1540 NMDA-Mediated Regulation of DSCAM Dendritic Local Translation Is Lost in a Mouse Model of Down's Syndrome
Alves-Sampaio, A., Troca-Marin, J.A. and Montesinos, M.L.
J. Neurosci., ***30(40)***, *13537-13548 (2010)*

Down's syndrome cell adhesion molecule (*DSCAM*) belongs to the Down's syndrome critical region of human chromosome 21, and it encodes a cell adhesion molecule involved in dendrite morphology and neuronal wiring. Although the function of *DSCAM* in the adult brain is unknown, its expression pattern suggests a role in synaptic plasticity. Local mRNA translation is a key process in axonal growth, dendritogenesis, and synaptogenesis during development, and in synaptic plasticity in adulthood. Here, we report the dendritic localization of *DSCAM* mRNA in the adult mouse hippocampus, where it associates with CPEB1 [cytoplasmic polyadenylation element (CPE) binding protein 1], an important regulator of mRNA transport and local translation. We identified five *DSCAM* isoforms produced by alternative polyadenylation bearing different combinations of regulatory CPE motifs. Overexpression of DSCAM in hippocampal neurons inhibited dendritic branching. Interestingly, dendritic levels of *DSCAM* mRNA and protein were increased in hippocampal neurons from Ts1Cje mice, a model of Down's syndrome. Most importantly, *DSCAM* dendritic translation was rapidly induced by NMDA in wild-type, but not in Ts1Cje neurons. We propose that impairment of the NMDA-mediated regulation of *DSCAM* translation may contribute to the alterations in dendritic morphology and/or synaptic plasticity in Down's syndrome.

3.1541 Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis
Heaton, N.S., Perera, R., Berger, K.L., Khadka, S., LaCount, D.J., Kuhn, R.J. and Randall, G.
PNAS, ***107(40)***, *17345-17350 (2010)*

Dengue virus (DENV) modifies cellular membranes to establish its sites of replication. Although the 3D architecture of these structures has recently been described, little is known about the cellular pathways required for their formation and expansion. In this report, we examine the host requirements for DENV replication using a focused RNAi analysis combined with validation studies using pharmacological inhibitors. This approach identified three cellular pathways required for DENV replication: autophagy, actin polymerization, and fatty acid biosynthesis. Further characterization of the viral modulation of fatty acid biosynthesis revealed that a key enzyme in this pathway, fatty acid synthase (FASN), is relocalized to sites of DENV replication. DENV nonstructural protein 3 (NS3) is responsible for FASN recruitment, inasmuch as (*i*) NS3 expressed in the absence of other viral proteins colocalizes with FASN and (*ii*) NS3 interacts with FASN in a two-hybrid assay. There is an associated increase in the rate of fatty acid biosynthesis in DENV-infected cells, and de novo synthesized lipids preferentially cofractionate with DENV RNA. Finally, purified recombinant NS3 stimulates the activity of FASN in vitro. Taken together, these experiments suggest that DENV co-opts the fatty acid biosynthetic pathway to establish its replication complexes. This study provides mechanistic insight into DENV membrane remodeling and highlights the potential for the development of therapeutics that inhibit DENV replication by targeting the fatty acid biosynthetic pathway.

3.1542 Organelle Proteomics by Label-Free and SILAC-Based Protein Correlation Profiling
Dengjel, J. Jakobsen, L. and Andersen, J.S.
Methods in Mol. Biol., ***658***, *255-265 (2010)*

The ability to purify cell organelles and protein complexes on a large scale, combined with advances in protein identification using mass spectrometry, has provided a wealth of information regarding protein localization and function. A major challenge in these studies has been the ability to identify bona fide organelle components from a background of co-purifying contaminants because none of the available biochemical purification protocols afford pure preparations. Since this situation is unlikely to change alternative strategies have been devised to meet this challenge by making use of the information inherent in the fractionation profile of organelles isolated by density gradient centrifugation. In this chapter we describe strategies based on protein correlation profiling and quantitative mass spectrometry to sort out likely candidates. The organelle inventories defined by these methods are suitable to guide future functional experiments.

3.1543 Critical role of lipid rafts in virus entry and activation of phosphoinositide 3′ kinase/Akt signaling during early stages of Japanese encephalitis virus infection in neural stem/progenitor cells
Das, S., Chakraborty, S. and Basu, A.
J. Neurochem., ***115***, *537-549 (2010)*

Japanese encephalitis virus (JEV), the leading cause of acute encephalitis in South-East Asia is a neurotropic virus infecting various CNS cell types. Most Flaviviruses including JEV get internalised into cells by receptor-mediated endocytosis, which involve clathrin and membrane cholesterol. The cholesterol-enriched membrane microdomains referred to as lipid rafts act as portals for virus entry in a number of enveloped viruses, including Flavivirus. However, the precise role played by membrane lipid rafts in JEV internalisation into neural stem cells is still unknown. We have established neural stem/progenitor cells and C17.2 cell line as models of productive JEV infection. Increase in membrane fluidity and clustering of viral envelope proteins in lipid rafts was observed in early time points of infection. Localisation of non-structural proteins to rafts at later infection stages was also observed. Co-localisation of JEV glycoprotein with Cholera toxin B confirmed that JEV internalisation occurs in a lipid-raft dependent manner. Though JEV entry is raft dependent, however, there is requirement of functional clathrin during endocytosis inside the cells. Besides virus entry, the lipid rafts act as signalling platforms for Src tyrosine kinases and result in activation of phosphoinositide 3′-kinase/Akt signalling during early JEV infection. Disruption of lipid raft formation by cholesterol depletion using Methyl β-cyclodextrin, reduced JEV RNA levels and production of infectious virus particles as well as impaired phosphoinositide 3′-kinase/Akt signalling during initial infection. Overall, our results implicate the importance of host membrane lipid rafts in JEV entry and life cycle, besides maintaining survival of neural stem/progenitor cells during early infection.

3.1544 Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol
Zhu, X., Owen, J.S., Wilson, M.D., Li, H., Griffiths, G.L., Thomas, M.J., Hiltbold, E.M., Fessler, M.B. and Parks, J.S.
J. Lipid Res., ***51***, *3196-3206 (2010)*

We previously showed that macrophages from macrophage-specific ATP-binding cassette transporter A1 (ABCA1) knockout (*Abca1*$^{-M/-M}$) mice had an enhanced proinflammatory response to the Toll-like receptor (TLR) 4 agonist, lipopolysaccharide (LPS), compared with wild-type (WT) mice. In the present study, we demonstrate a direct association between free cholesterol (FC), lipid raft content, and hyper-responsiveness of macrophages to LPS in WT mice. *Abca1*$^{-M/-M}$ macrophages were also hyper-responsive to specific agonists to TLR2, TLR7, and TLR9, but not TLR3, compared with WT macrophages. We hypothesized that ABCA1 regulates macrophage responsiveness to TLR agonists by modulation of lipid raft cholesterol and TLR mobilization to lipid rafts. We demonstrated that *Abca1*$^{-M/-M}$ vs. WT macrophages contained 23% more FC in isolated lipid rafts. Further, mass spectrometric analysis suggested raft phospholipid composition was unchanged. Although cell surface expression of TLR4 was similar between *Abca1*$^{-M/-M}$ and WT macrophages, significantly more TLR4 was distributed in membrane lipid rafts in *Abca1*$^{-M/-M}$ macrophages. *Abca1*$^{-M/-M}$ macrophages also exhibited increased trafficking of the predominantly intracellular TLR9 into lipid rafts in response to TLR9-specific agonist (CpG). Collectively, our data suggest that macrophage ABCA1 dampens inflammation by reducing MyD88-dependent TLRs trafficking to lipid rafts by selective reduction of FC content in lipid rafts.

3.1545 Biogenesis of Salmonella enterica Serovar Typhimurium Membrane Vesicles Provoked by Induction of PagC
Kitagawa, R., Takaya, A., Ohya, M., Mizunoe, Y., Takade, A., Yoshida, S-i., Isogai, E. and Yamamoto, T.
J: Bacteriol., ***192(21)***, *5645-5656 (2010)*

Gram-negative bacteria ubiquitously release membrane vesicles (MVs) into the extracellular milieu. Although MVs are the product of growing bacteria, not of cell lysis or death, the regulatory mechanisms underlying MV formation remained unknown. We have found that MV biogenesis is provoked by the induction of PagC, a *Salmonella*-specific protein whose expression is activated by conditions that mimic acidified macrophage phagosomes. PagC is a major constituent of *Salmonella* MVs, and increased expression accelerates vesiculation. Expression of PagC is regulated at the posttranscriptional and/or posttranslational level in a sigmaS (RpoS)-dependent manner. Serial quantitative analysis has demonstrated that MV formation can accelerate when the quantity of the MV constituents, OmpX and PagC, rises. Overproduction of PagC dramatically impacts the difference in the relative amount of vesiculation, but the corresponding overproduction of OmpX was less pronounced. Quantitative examination of the ratios of

PagC and OmpX in the periplasm, outer membrane, and MVs demonstrates that PagC is preferentially enriched in MVs released from *Salmonella* cells. This suggests that specific protein sorting mechanisms operate when MVs are formed. The possible role(s) of PagC-MV in host cells is discussed.

3.1546 Relevant Elements of a Maize γ-Zein Domain Involved in Protein Body Biogenesis
Llop-Tous, I., Madurga, S., giralt, E., Torrent, M. and Dolors-Ludevid, M.
J. Biol. Chem., ***285(46)***, *35633-35644 (2010)*

The N-terminal proline-rich domain of γ-zein (Zera) plays an important role in protein body (PB) formation not only in the original host (maize seeds) but in a broad spectrum of eukaryotic cells. However, the elements within the Zera sequence that are involved in the biogenesis of PBs have not been clearly identified. Here, we focused on amino acid sequence motifs that could be involved in Zera oligomerization, leading to PB-like structures in *Nicotiana benthamiana* leaves. By using fusions of Zera with fluorescent proteins, we found that the lack of the repeat region $(PPPVHL)_8$ of Zera resulted in the secretion of the fusion protein but that this repeat by itself did not form PBs. Although the repeat region containing eight units was the most efficient for Zera self-assembly, shorter repeats of 4–6 units still formed small multimers. Based on site-directed mutagenesis of Zera cysteine residues and analysis of multimer formation, we conclude that the two N-terminal Cys residues of Zera (Cys^7 and Cys^9) are critical for oligomerization. Immunoelectron microscopy and confocal studies on PB development over time revealed that early, small, Zera-derived oligomers were sequestered in buds along the rough ER and that the mature size of the PBs could be attained by both cross-linking of preformed multimers and the incorporation of new chains of Zera fusions synthesized by active membrane-bound ribosomes. Based on these results and on the behavior of the Zera structure determined by molecular dynamics simulation studies, we propose a model of Zera-induced PB biogenesis.

3.1547 Protein-disulfide Isomerase-associated 3 (Pdia3) Mediates the Membrane Response to 1,25-Dihydroxyvitamin D3 in Osteoblasts
Chen, J., Olivares-Navarrete, R., Wang, Y., Herman, T.R., Boyan, B.D. and Schwartz, Z.
J. Biol. Chem., ***285(47)***, *37041-37050 (2010)*

Protein-disulfide isomerase-associated 3 (Pdia3) is a multifunctional protein hypothesized to be a membrane receptor for 1,25(OH)2D3. In intestinal epithelium and chondrocytes, 1,25(OH)2D3 stimulates rapid membrane responses that are different from genomic effects via the vitamin D receptor (VDR). In this study, we show that 1,25(OH)2D3 stimulates phospholipase A2 (PLA2)-dependent rapid release of prostaglandin E2 (PGE2), activation of protein kinase C (PKC), and regulation of bone-related gene transcription and mineralization in osteoblast-like MC3T3-E1 cells (WT) via a mechanism involving Pdia3. Pdia3 was present in caveolae based on co-localization with lipid rafts and caveolin-1. In Pdia3-silenced (Sh-Pdia3) cells, 1,25(OH)2D3 failed to stimulate PKC and PGE2 responses; in Pdia3-overexpressing cells (Ov-Pdia3), responses to 1,25(OH)2D3 were augmented. Downstream mediators of Pdia3, PLA2-activating protein (PLAA) and arachidonic acid, stimulated similar PKC activation in wild-type, Sh-Pdia3, and Ov-Pdia3 cells supporting the hypothesis that Pdia3 mediates the membrane action of 1,25(OH)2D3. Treatment of MC3T3-E1 cells with 1,25(OH)2D3 for 9 min stimulated rapid phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and increased expression of alkaline phosphatase, MMP-13, and osteopontin but decreased expression of osteocalcin, osteoprotegerin (mRNA and protein), and smad2. These effects were attenuated in Sh-Pdia3 cells. Sh-Pdia3 cells produced higher numbers of von Kossa-positive nodules and alizarin red-positive nodules compared with WT cells with or without 1,25(OH)2D3 treatment whereas Ov-Pdia3 did not show any mineralization. Our data suggest Pdia3 is an important initiator of 1,25(OH)2D3-stimulated membrane signaling pathways, which have both genomic and non genomic effects during osteoblast maturation.

3.1548 Helicobacter pylori Exploits Cholesterol-Rich Microdomains for Induction of NF-κB-Dependent Responses and Peptidoglycan Delivery in Epithelial Cells
Hutton, M.L., Kaparakis-Liaskos, M., Turner, L., cardona, A., Kwok, T. and Ferrero, R.L.
Infect. Immun., ***78(11)***

Infection with Helicobacter pylori cag pathogenicity island (cagPAI)-positive strains is associated with more destructive tissue damage and an increased risk of severe disease. The cagPAI encodes a type IV secretion system (TFSS) that delivers the bacterial effector molecules CagA and peptidoglycan into the host cell cytoplasm, thereby inducing responses in host cells. It was previously shown that interactions between CagL, present on the TFSS pilus, and host α5β1 integrin molecules were critical for CagA

translocation and the induction of cytoskeletal rearrangements in epithelial cells. As the α5β1 integrin is found in cholesterol-rich microdomains (known as lipid rafts), we hypothesized that these domains may also be involved in the induction of proinflammatory responses mediated by NOD1 recognition of H. pylori peptidoglycan. Indeed, not only did methyl-β-cyclodextrin depletion of cholesterol from cultured epithelial cells have a significant effect on the levels of NF-κB and interleukin-8 (IL-8) responses induced by H. pylori bacteria with an intact TFSS ($P < 0.05$), but it also interfered with TFSS-mediated peptidoglycan delivery to cells. Both of these effects could be restored by cholesterol replenishment of the cells. Furthermore, we demonstrated for the first time the involvement of α5β1 integrin in the induction of proinflammatory responses by H. pylori. Taking the results together, we propose that α5β1 integrin, which is associated with cholesterol-rich microdomains at the host cell surface, is required for NOD1 recognition of peptidoglycan and subsequent induction of NF-κB-dependent responses to H. pylori. These data implicate cholesterol-rich microdomains as a novel platform for TFSS-dependent delivery of bacterial products to cytosolic pathogen recognition molecules.

3.1549 Dynamin-like protein 1 at the Golgi complex: A novel component of the sorting/targeting machinery en route to the plasma membrane
Bomekamp, N.A., Vormund, K., Jacob, R. and Schrader, M.
Exp. Cell Res., ***316(20)***, *3454-3467 (2010)*

The final step in the liberation of secretory vesicles from the trans-Golgi network (TGN) involves the mechanical action of the large GTPase dynamin as well as conserved dynamin-independent fission mechanisms, e.g. mediated by Brefeldin A-dependent ADP-ribosylated substrate (BARS). Another member of the dynamin family is the mammalian dynamin-like protein 1 (DLP1/Drp1) that is known to constrict and tubulate membranes, and to divide mitochondria and peroxisomes. Here, we examined a potential role for DLP1 at the Golgi complex. DLP1 localized to the Golgi complex in some but not all cell lines tested, thus explaining controversial reports on its cellular distribution. After silencing of DLP1, an accumulation of the apical reporter protein YFP-GL-GPI, but not the basolateral reporter VSVG-SP-GFP at the Golgi complex was observed. A reduction in the transport of YFP-GL-GPI to the plasma membrane was confirmed by surface immunoprecipitation and TGN-exit assays. In contrast, YFP-GL-GPI trafficking was not disturbed in cells silenced for BARS, which is involved in basolateral sorting and trafficking of VSVG-SP-GFP in COS-7 cells. Our data indicate a new role for DLP1 at the Golgi complex and thus a role for DLP1 as a novel component of the apical sorting machinery at the TGN is discussed.

3.1550 Visfatin-induced lipid raft redox signaling platforms and dysfunction in glomerular endothelial cells
Boini, K.M., Zhang, C., Xia, M., Han, W-Q., Brimson, C., Poklis, J.L. and Li, P-l.
Biochim. Biophys. Acta, ***1801***, *1294-1304 (2010)*

Adipokines have been reported to contribute to glomerular injury during obesity or diabetes mellitus. However, the mechanisms mediating the actions of various adipokines on the kidney remained elusive. The present study was performed to determine whether acid sphingomyelinase (ASM)-ceramide associated lipid raft (LR) clustering is involved in local oxidative stress in glomerular endothelial cells (GECs) induced by adipokines such as visfatin and adiponectin. Using confocal microscopy, visfatin but not adiponectin was found to increase LRs clustering in the membrane of GECs in a dose and time dependent manner. Upon visfatin stimulation ASMase activity was increased, and an aggregation of ASMase product, ceramide and NADPH oxidase subunits, $gp91^{phox}$ and $p47^{phox}$ was observed in the LR clusters, forming a LR redox signaling platform. The formation of this signaling platform was blocked by prior treatment with LR disruptor filipin, ASMase inhibitor amitriptyline, ASMase siRNA, $gp91^{phox}$ siRNA and adiponectin. Corresponding to LR clustering and aggregation of NADPH subunits, superoxide ($O_2{\bullet}^-$) production was significantly increased (2.7 folds) upon visfatin stimulation, as measured by electron spin resonance (ESR) spectrometry. Functionally, visfatin significantly increased the permeability of GEC layer in culture and disrupted microtubular networks, which were blocked by inhibition of LR redox signaling platform formation. In conclusion, the injurious effect of visfatin, but not adiponectin on the glomerular endothelium is associated with the formation of LR redox signaling platforms via LR clustering, which produces local oxidative stress resulting in the disruption of microtubular networks in GECs and increases the glomerular permeability.

3.1551 Synphilin-1 Enhances α-Synuclein Aggregation in Yeast and Contributes to Cellular Stress and Cell Death in a Sir2-Dependent Manner

Büttner, S., Delay, C., Franssens, V., Bammens, T., Ruli, D., Zaunschirm, S., Machado de oliveira, R., Outeiro, T.F., Madeo, F., Buee, L., Galas, M-C. and Winderickx, J.
*PloSONe, **5(10)**, e13700 (2010)*

Background
Parkinson's disease is characterized by the presence of cytoplasmic inclusions, known as Lewy bodies, containing both aggregated α-synuclein and its interaction partner, synphilin-1. While synphilin-1 is known to accelerate inclusion formation by α-synuclein in mammalian cells, its effect on cytotoxicity remains elusive.
Methodology/Principal Findings
We expressed wild-type synphilin-1 or its R621C mutant either alone or in combination with α-synuclein in the yeast Saccharomyces cerevisiae and monitored the intracellular localization and inclusion formation of the proteins as well as the repercussions on growth, oxidative stress and cell death. We found that wild-type and mutant synphilin-1 formed inclusions and accelerated inclusion formation by α-synuclein in yeast cells, the latter being correlated to enhanced phosphorylation of serine-129. Synphilin-1 inclusions co-localized with lipid droplets and endomembranes. Consistently, we found that wild-type and mutant synphilin-1 interacts with detergent-resistant membrane domains, known as lipid rafts. The expression of synphilin-1 did not incite a marked growth defect in exponential cultures, which is likely due to the formation of aggresomes and the retrograde transport of inclusions from the daughter cells back to the mother cells. However, when the cultures approached stationary phase and during subsequent ageing of the yeast cells, both wild-type and mutant synphilin-1 reduced survival and triggered apoptotic and necrotic cell death, albeit to a different extent. Most interestingly, synphilin-1 did not trigger cytotoxicity in ageing cells lacking the sirtuin Sir2. This indicates that the expression of synphilin-1 in wild-type cells causes the deregulation of Sir2-dependent processes, such as the maintenance of the autophagic flux in response to nutrient starvation.
Conclusions/Significance
Our findings demonstrate that wild-type and mutant synphilin-1 are lipid raft interacting proteins that form inclusions and accelerate inclusion formation of α-synuclein when expressed in yeast. Synphilin-1 thereby induces cytotoxicity, an effect most pronounced for the wild-type protein and mediated via Sir2-dependent processes.

3.1552 The Translocon Sec61β Localized in the Inner Nuclear Membrane Transports Membrane-embedded EGF Receptor to the Nucleus

Wang, Y-N., Yamaguchi, H., Huo, L., Lee, H-J., Lee, H-H., Wang, H., Hsu, J-M. and Hung, M-C.
*J. Biol. Chem., **285(49)**, 38720-38729 (2010)*

Accumulating evidence indicates that endocytosis plays an essential role in the nuclear transport of the ErbB family members, such as epidermal growth factor receptor (EGFR) and ErbB-2. Nevertheless, how full-length receptors embedded in the endosomal membrane pass through the nuclear pore complexes and function as non-membrane-bound receptors in the nucleus remains unclear. Here we show that upon EGF treatment, the biotinylated cell surface EGFR is trafficked to the inner nuclear membrane (INM) through the nuclear pore complexes, remaining in a membrane-bound environment. We further find that importin β regulates EGFR nuclear transport to the INM in addition to the nucleus/nucleoplasm. Unexpectedly, the well known endoplasmic reticulum associated translocon Sec61β is found to reside in the INM and associate with EGFR. Knocking down Sec61β expression reduces EGFR level in the nucleoplasm portion and accumulates it in the INM portion. Thus, the Sec61β translocon plays an unrecognized role in the release of the membrane-anchored EGFR from the lipid bilayer of the INM to the nucleus. The newly identified Sec61β function provides an alternative pathway for nuclear transport that can be utilized by membrane-embedded proteins such as full-length EGFR.

3.1553 MAL/VIP17, a New Player in the Regulation of NKCC2 in the Kidney

Carmosino, M., Rizzo, F., Procino, G., Basco, D., Valenti, G., Forbush, B., Schaere-Wiemers, N., Caplan, M.J. and Svelto, M.
*Mol. Biol. Cell, **21**, 3985-3997 (2010)*

The renal-specific Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) is the major salt transport pathway of the apical membrane of the mammalian thick ascending limb of Henle's loop. Here, we analyze the role of the tetraspan protein myelin and lymphocytes-associated protein (MAL)/VIP17 in the regulation of NKCC2.

We demonstrated that 1) NKCC2 and MAL/VIP17 colocalize and coimmunoprecipitate in Lilly Laboratories cell porcine kidney cells (LLC-PK1) as well as in rat kidney medullae, 2) a 150-amino acid stretch of NKCC2 C-terminal tail is involved in the interaction with MAL/VIP17, 3) MAL/VIP17 increases the cell surface retention of NKCC2 by attenuating its internalization, and 4) this coincides with an increase in cotransporter phosphorylation. Interestingly, overexpression of MAL/VIP17 in the kidney of transgenic mice results in cysts formation in distal nephron structures consistent with the hypothesis that MAL/VIP17 plays an important role in apical sorting or in maintaining the stability of the apical membrane. The NKCC2 expressed in these mice was highly glycosylated and phosphorylated, suggesting that MAL/VIP17 also is involved in the stabilization of NKCC2 at the apical membrane in vivo. Thus, the involvement of MAL/VIP17 in the activation and surface expression of NKCC2 could play an important role in the regulated absorption of Na^+ and Cl^- in the kidney.

3.1554 Abl Tyrosine Kinase Phosphorylates Nonmuscle Myosin Light Chain Kinase to Regulate Endothelial Barrier Function

Dudek, S.M., Chiang, E.T., Camp, S.M., Guo, Y., Zhao, J., Brown, M.E., Singleton, P.A., Wang, L., Desai, A., Arce, F.T., Lal, R., Van Eyk, J.E., Imam, S.Z. and Garcia, J.G.N.
Mol. Biol. Cell, ***21***, *4042-4056 (2010)*

Nonmuscle myosin light chain kinase (nmMLCK), a multi-functional cytoskeletal protein critical to vascular homeostasis, is highly regulated by tyrosine phosphorylation. We identified multiple novel c-Abl–mediated nmMLCK phosphorylation sites by mass spectroscopy analysis (including Y^{231}, Y^{464}, Y^{556}, Y^{846}) and examined their influence on nmMLCK function and human lung endothelial cell (EC) barrier regulation. Tyrosine phosphorylation of nmMLCK increased kinase activity, reversed nmMLCK-mediated inhibition of Arp2/3-mediated actin polymerization, and enhanced binding to the critical actin-binding phosphotyrosine protein, cortactin. EC challenge with sphingosine 1-phosphate (S1P), a potent barrier-enhancing agonist, resulted in c-Abl and phosphorylated nmMLCK recruitment into caveolin-enriched microdomains, rapid increases in Abl kinase activity, and spatial targeting of c-Abl to barrier-promoting cortical actin structures. Conversely, reduced c-Abl expression in EC (siRNA) markedly attenuated S1P-mediated cortical actin formation, reduced the EC modulus of elasticity (assessed by atomic force microscopy), reduced nmMLCK and cortactin tyrosine phosphorylation, and attenuated S1P-mediated barrier enhancement. These studies indicate an essential role for Abl kinase in vascular barrier regulation via posttranslational modification of nmMLCK and strongly support c-Abl-cortactin-nmMLCK interaction as a novel determinant of cortical actin-based cytoskeletal rearrangement critical to S1P-mediated EC barrier enhancement.

3.1555 High-molecular-weight hyaluronan is a novel inhibitor of pulmonary vascular leakiness

Singleton, P.AQ., Mirzapoiazova, T., Guo, Y., Sammani, S., Mambetsariev, N., Lennon, F.E., Moreno-Vinasco, L. and Garcia, J.G.N.
Am. J. Physiol. Lung Cell. Mol. Physiol., ***299***, *L639-L651 (2010)*

Endothelial cell (EC) barrier dysfunction results in increased vascular permeability, a perturbation observed in inflammatory states, tumor angiogenesis, atherosclerosis, and both sepsis and acute lung injury. Therefore, agents that enhance EC barrier integrity have important therapeutic implications. We observed that binding of high-molecular-weight hyaluronan (HMW-HA) to its cognate receptor CD44 within caveolin-enriched microdomains (CEM) enhances human pulmonary EC barrier function. Immunocytochemical analysis indicated that HMW-HA promotes redistribution of a significant population of CEM to areas of cell-cell contact. Quantitative proteomic analysis of CEM isolated from human EC demonstrated HMW-HA-mediated recruitment of cytoskeletal regulatory proteins (annexin A2, protein S100-A10, and filamin A/B). Inhibition of CEM formation [caveolin-1 small interfering RNA (siRNA) and cholesterol depletion] or silencing (siRNA) of CD44, annexin A2, protein S100-A10, or filamin A/B expression abolished HMW-HA-induced actin cytoskeletal reorganization and EC barrier enhancement. To confirm our in vitro results in an in vivo model of inflammatory lung injury with vascular hyperpermeability, we observed that the protective effects of HMW-HA on LPS-induced pulmonary vascular leakiness were blocked in caveolin-1 knockout mice. Furthermore, targeted inhibition of CD44 expression in the mouse pulmonary vasculature significantly reduced HMW-HA-mediated protection from LPS-induced hyperpermeability. These data suggest that HMW-HA, via CD44-mediated CEM signaling events, represents a potentially useful therapeutic agent for syndromes of increased vascular permeability.

3.1556 Shank2 redistributes with NaPilla during regulated endocytosis
Dobrinskikh, E., Giral, H., Caldas, Y.A., Levi, M and Doctor, R.B.
Am. J. Physiol. Cell Physiol., ***299***, *C1324-C1334 (2010)*

Serum phosphate levels are acutely impacted by the abundance of sodium-phosphate cotransporter IIa (NaPiIIa) in the apical membrane of renal proximal tubule cells. PSD-95/Disks Large/Zonula Occludens (PDZ) domain-containing proteins bind NaPiIIa and likely contribute to the delivery, retention, recovery, and trafficking of NaPiIIa. Shank2 is a distinctive PDZ domain protein that binds NaPiIIa. Its role in regulating NaPiIIa activity, distribution, and abundance is unknown. In the present in vivo study, rats were maintained on a low-phosphate diet, and then plasma phosphate levels were acutely elevated by high-phosphate feeding to induce the recovery, endocytosis, and degradation of NaPiIIa. Western blot analysis of renal cortical tissue from rats given high-phosphate feed showed NaPiIIa and Shank2 underwent degradation. Quantitative immunofluorescence analyses, including microvillar versus intracellular intensity ratios and intensity correlation quotients, showed that Shank2 redistributed with NaPiIIa during the time course of NaPiIIa endocytosis. Furthermore, NaPiIIa and Shank2 trafficked through distinct endosomal compartments (clathrin, early endosomes, lysosomes) with the same temporal pattern. These in vivo findings indicate that Shank2 is positioned to coordinate the regulated endocytic retrieval and downregulation of NaPiIIa in rat renal proximal tubule cells.

3.1557 Nuclear angiotensin-(1–7) receptor is functionally coupled to the formation of nitric oxide
Gwatmey, T.M., Westwood, B.M., Pirro, N.T., Tang, L., Rose, J.C., Diz, D.I. and Chappell, M.C.
Am. J. Physiol. Renal Physiol., ***299***, *F983-F990 (2010)*

The kidney is an important target for the actions of the renin-angiotensin system (RAS) and this tissue contains a complete local RAS that expresses the bioactive peptides angiotensin II (ANG II) and Ang-(1–7). We find both angiotensin type 1 (AT_1R) and type 2 (AT_2R) receptors expressed on renal nuclei that stimulate reactive oxygen species and nitric oxide (NO), respectively. Since Ang-(1–7) also exhibits actions within the kidney and the Ang-(1–7)/Mas receptor protein contains a nuclear localization sequence, we determined the expression of Ang-(1–7) receptors in nuclei isolated from the kidneys of young adult sheep. Binding studies with ^{125}I-[Sar^{1}Thr^8]-ANG II revealed sites sensitive to the Ang-(1–7) antagonist [D-Ala^7]-Ang-(1–7) (DALA, A779), as well as to AT_2 and AT_1 antagonists. Incubation of Ang-(1–7) [10^{-15} to 10^{-9} M] with isolated cortical nuclei elicited a dose-dependent increase in the fluorescence of the NO indicator [4-amino-5-methylamino-2',7']-difluorofluorescein diacetate. The NO response to Ang-(1–7) was abolished by the NO inhibitor *N*-nitro-L-arginine methyl ester and DALA, but not the AT_1 antagonist losartan or the AT_2 blocker PD123319. Immunofluorescent studies utilizing the Ang-(1–7)/Mas receptor antibody revealed immunolabeling of the proximal tubules but not staining within the glomerulus in cortical sections of the sheep kidney. In the nuclear fraction of isolated proximal tubules, immunoblots revealed the precursor angiotensinogen and renin, as well as functional activity for ACE, ACE2, and neprilysin. We conclude that renal nuclei express Ang-(1–7)/Mas receptors that are functionally linked to NO formation. The marked sensitivity of the intracellular NO response to Ang-(1–7) implicates a functional role of the Ang-(1–7) axis within the nucleus. Moreover, evidence for the precursor and enzymatic components of the RAS within the nuclear compartment of the proximal tubules provides a potential pathway for the intracellular generation of Ang-(1–7).

3.1558 The Huntington's disease mutation impairs Huntingtin's role in the transport of NF-κB from the synapse to the nucleus
Marcora, E. and Kennedy, M.B.
Human Mol. Genet., ***19(22)***, *4373-4384 (2010)*

Expansion of a polyglutamine (polyQ) tract in the Huntingtin (Htt) protein causes Huntington's disease (HD), a fatal inherited neurodegenerative disorder. Loss of the normal function of Htt is thought to be an important pathogenetic component of HD. However, the function of wild-type Htt is not well defined. Htt is thought to be a multifunctional protein that plays distinct roles in several biological processes, including synaptic transmission, intracellular transport and neuronal transcription. Here, we show with biochemical and live cell imaging studies that wild-type Htt stimulates the transport of nuclear factor κ light-chain-enhancer of activated B cells (NF-κB) out of dendritic spines (where NF-κB is activated by excitatory synaptic input) and supports a high level of active NF-κB in neuronal nuclei (where NF-κB stimulates the transcription of target genes). We show that this novel function of Htt is impaired by the polyQ expansion and thus may contribute to the etiology of HD.

3.1559 Epithelial septate junction assembly relies on melanotransferrin iron binding and endocytosis in *Drosophila*

Tiklova, K., Senti, K-A., Wang, S., Gräslund, A. and Samakovlis, C.
Nature Cell. Biol., ***12(11)***, *1071-1078 (2010)*

Iron is an essential element in many biological processes. In vertebrates, serum transferrin is the major supplier of iron to tissues, but the function of additional transferrin-like proteins remains poorly understood. Melanotransferrin (MTf) is a phylogenetically conserved, iron-binding epithelial protein. Elevated MTf levels have been implicated in melanoma pathogenesis. Here, we present a functional analysis of MTf in *Drosophila melanogaster*. Similarly to its human homologue, *Drosophila* MTf is a lipid-modified, iron-binding protein attached to epithelial cell membranes, and is a component of the septate junctions that form the paracellular permeability barrier in epithelial tissues. We demonstrate that septate junction assembly during epithelial maturation relies on endocytosis and apicolateral recycling of iron-bound MTf. Mouse *MTf* complements the defects of *Drosophila MTf* mutants. *Drosophila* provides the first genetic model for the functional dissection of MTf in epithelial junction assembly and morphogenesis.

3.1560 Oxidative stress increases blood–brain barrier permeability and induces alterations in occludin during hypoxia–reoxygenation

Lochhead, J.J., McCaffrey, G., Quigley, C.E., Finch, J., DeMarco, K.M., Nametz, N. and Davis, T.P.
J. Cerebral Blood Flow & Metab., ***30***, *1625-1636 (2010)*

The blood–brain barrier (BBB) has a critical role in central nervous system homeostasis. Intercellular tight junction (TJ) protein complexes of the brain microvasculature limit paracellular diffusion of substances from the blood into the brain. Hypoxia and reoxygenation (HR) is a central component to numerous disease states and pathologic conditions. We have previously shown that HR can influence the permeability of the BBB as well as the critical TJ protein occludin. During HR, free radicals are produced, which may lead to oxidative stress. Using the free radical scavenger tempol (200 mg/kg, intraperitoneal), we show that oxidative stress produced during HR (6% O_2 for 1 h, followed by room air for 20 min) mediates an increase in BBB permeability in vivo using in situ brain perfusion. We also show that these changes are associated with alterations in the structure and localization of occludin. Our data indicate that oxidative stress is associated with movement of occludin away from the TJ. Furthermore, subcellular fractionation of cerebral microvessels reveals alterations in occludin oligomeric assemblies in TJ associated with plasma membrane lipid rafts. Our data suggest that pharmacological inhibition of disease states with an HR component may help preserve BBB functional integrity.

3.1561 Cholesterol Lipids of Borrelia burgdorferi Form Lipid Rafts and Are Required for the Bactericidal Activity of a Complement-Independent Antibody

LaRocca, T.J., Crowley, J.T., Cusack, B.J., Pathak, P., Benach, J., London, E., Garcia-Monco, J.C. and Benach, J.L.
Cell Host & Microbe, ***8(4)***, *331-342 (2010)*

Borrelia burgdorferi, the agent of Lyme disease, is unusual as it contains free cholesterol and cholesterol glycolipids. It is also susceptible to complement-independent bactericidal antibodies, such as CB2, a monoclonal IgG1 against outer surface protein B (OspB). We find that the bactericidal action of CB2 requires the presence of cholesterol glycolipids and cholesterol. Ultrastructural, biochemical, and biophysical analysis revealed that the bacterial cholesterol glycolipids exist as lipid raft-like microdomains in the outer membrane of cultured and mouse-derived *B. burgdorferi* and in model membranes from *B. burgdorferi* lipids. The order and size of the microdomains are temperature sensitive and correlate with the bactericidal activity of CB2. This study demonstrates the existence of cholesterol-containing lipid raft-like microdomains in a prokaryote, and we suggest that the temperature dependence of *B. burgdorferi* lipid raft organization may have significant implications in the transmission cycle of the spirochetes which are exposed to a range of temperatures.

3.1562 Extensive sphingolipid depletion does not affect lipid raft integrity or lipid raft localization and efflux function of the ABC transporter MRP1

Klappe, K., Dijkhuis, A-J., Hummel, I., van Dam, A., Ivanova, P.T., Milne, S.B., Myers, D.S., Brown, H.A., Permentier, H. and Kok, J.W.

Biochem. J., ***430***, *519-529 (2010)*

We show that highly efficient depletion of sphingolipids in two different cell lines does not abrogate the ability to isolate Lubrol-based DRMs (detergent-resistant membranes) or detergent-free lipid rafts from these cells. Compared with control, DRM/detergent-free lipid raft fractions contain equal amounts of protein, cholesterol and phospholipid, whereas the classical DRM/lipid raft markers Src, caveolin-1 and flotillin display the same gradient distribution. DRMs/detergent-free lipid rafts themselves are severely depleted of sphingolipids. The fatty acid profile of the remaining sphingolipids as well as that of the glycerophospholipids shows several differences compared with control, most prominently an increase in highly saturated C_{16} species. The glycerophospholipid headgroup composition is unchanged in sphingolipid-depleted cells and cell-derived detergent-free lipid rafts. Sphingolipid depletion does not alter the localization of MRP1 (multidrug-resistance-related protein 1) in DRMs/detergent-free lipid rafts or MRP1-mediated efflux of carboxyfluorescein. We conclude that extensive sphingolipid depletion does not affect lipid raft integrity in two cell lines and does not affect the function of the lipid-raft-associated protein MRP1.

3.1563 Dilysine retrieval signal-containing p24 proteins collaborate in inhibiting γ-cleavage of amyloid precursor protein

Hasegawa, H., Liu, L. and Nishimura, M.

J. Neurochem., ***115(3)***, *771-781 (2010)*

γ-Secretase mediates intramembranous γ-cleavage and ε-cleavage of β-amyloid precursor protein (APP) to liberate β-amyloid peptide (Aβ) and APP intracellular domain respectively from the membrane. Although the regulatory mechanism of γ-secretase cleavage remains unresolved, a member of the p24 cargo protein family, named $p24\delta_1$ or TMP21, has been identified as an activity-modulating component. The p24 family proteins are divided into four subfamilies (p24α, β, δ and γ). In contrast to $p24\delta_1$, $p24\beta_1$ has reportedly no effect on γ-cleavage. In this study, we determined whether $p24\alpha_2$, $p24\gamma_3$ or $p24\gamma_4$ modulates APP processing. Knockdown of cellular $p24\alpha_2$ induced a significant increase in Aβ generation but not in APP intracellular domain production in cell-based and cell-free assays, whereas $p24\alpha_2$ over-expression suppressed Aβ secretion. By contrast, Aβ secretion was not altered by $p24\gamma_3$ or $p24\gamma_4$ knockdown. Endogenous $p24\alpha_2$ co-immunoprecipitated with core components of the γ-secretase complex, and the anti-$p24\alpha_2$ immunoprecipitate exhibited γ-secretase activity. Mutational disruption of the conserved dilysine ER-retrieval motifs of $p24\alpha_2$ and $p24\delta_1$ perturbed inhibition of γ-cleavage. Simultaneous knockdown, or co-over-expression, of these proteins had no additive or synergistic effect on Aβ generation. Our findings suggest that dilysine ER-retrieval signal-containing p24 proteins, $p24\alpha_2$ and $p24\delta_1$, bind with γ-secretase complexes and collaborate in attenuating γ-cleavage of APP.

3.1564 Subcellular fractionation methods and strategies for proteomics

Lee, Y.H., Tan, H.T. and Chung, M.C.M.

Proteomics, ***10(22)***, *3935-3956 (2010)*

Developments in subcellular fractionation strategies have provided the means to profile and analyze the protein composition of organelles and cellular structures by proteomics. Here, we review the application of classical (*e.g.* density gradient centrifugation) and emerging sophisticated techniques (fluorescent-assisted organelle sorting) in the fractionation, and statistical/bioinformatics tools for the prediction of protein localization in subcellular proteomics. We also review the validation methods currently used (such as microscopy, RNA interference and multiple reaction monitoring) and discuss the importance of verification of the results obtained in subcellular proteomics. Finally, the numerous challenges facing subcellular proteomics including the dynamics of organelles are being examined. However, complementary approaches such as modern statistics, bioinformatics and large-scale integrative analysis are beginning to emerge as powerful tools to proteomics for analyzing subcellular organelles and structures.

3.1565 Organelle proteomics experimental designs and analysis

Gatto, L., Vizcaino, J.A., Hermjacob, H., Huber, W. and Lilley, K.S.

Proteomics, ***10(22)***, *3957-3969 (2010)*

In biology, localisation is function: knowledge of the localisation of proteins is of paramount importance to assess and study their function. This supports the need for reliable protein sub-cellular localisation assignment. Concomitant with recent technological advances in organelle proteomics, there is a requirement for more rigorous experimental and analysis design planning and description. In this review, we present an overview of current experimental designs in qualitative and quantitative organelle proteomics as well as associated data analysis. We also consider the major benefits associated with careful description and dissemination of the experiment and analysis designs, namely (i) comparison and optimisation of experimental designs and analysis pipelines, (ii) data validation, (iii) reproducible research, (iv) efficient repository submission and retrieval and (v) meta analysis. Formalisation of experimental design and analysis work flows is of direct benefit for the organelle proteomics researchers and will result in providing organelle localisation data of highest quality for the wider research community.

3.1566 Molecular characterization of the endoplasmic reticulum: Insights from proteomic studies
Chen, X., Karnovsky, A., Sans, M.D., Andrews, P.C. and Williams, J.A.
Proteomics, ***10(22)****, 4040-4052 (2010)*

The endoplasmic reticulum (ER) is a multifunctional intracellular organelle responsible for the synthesis, processing and trafficking of a wide variety of proteins essential for cell growth and survival. Therefore, comprehensive characterization of the ER proteome is of great importance to the understanding of its functions and has been actively pursued in the past decade by scientists in the proteomics field. This review summarizes major proteomic studies published in the past decade that focused on the ER proteome. We evaluate the data sets obtained from two different organs, liver and pancreas each of which contains a primary cell type (hepatocyte and acinar cell) with specialized functions. We also discuss how the nature of the proteins uncovered is related to the methods of organelle purification, organelle purity and the techniques used for protein separation prior to MS. In addition, this review also puts emphasis on the biological insights gained from these studies regarding the molecular functions of the ER including protein synthesis and translocation, protein folding and quality control, ER-associated degradation and ER stress, ER export and membrane trafficking, calcium homeostasis and detoxification and drug metabolism.

3.1567 The proteome of lysosomes
Schröder, B.A., Wrocklage, C., Hasilik, A. and Saftig, P.
Proteomics, ***10(22)****, 4053-4076 (2010)*

Lysosomes are organelles of eukaryotic cells that are critically involved in the degradation of macromolecules mainly delivered by endocytosis and autophagocytosis. Degradation is achieved by more than 60 hydrolases sequestered by a single phospholipid bilayer. The lysosomal membrane facilitates interaction and fusion with other compartments and harbours transport proteins catalysing the export of catabolites, thereby allowing their recycling. Lysosomal proteins have been addressed in various proteomic studies that are compared in this review regarding the source of material, the organelle/protein purification scheme, the proteomic methodology applied and the proteins identified. Distinguishing true constituents of an organelle from copurifying contaminants is a central issue in subcellular proteomics, with additional implications for lysosomes as being the site of degradation of many cellular and extracellular proteins. Although many of the lysosomal hydrolases were identified by classical biochemical approaches, the knowledge about the protein composition of the lysosomal membrane has remained fragmentary for a long time. Using proteomics many novel lysosomal candidate proteins have been discovered and it can be expected that their functional characterisation will help to understand functions of lysosomes at a molecular level that have been characterised only phenomenologically so far and to generally deepen our understanding of this indispensable organelle.

3.1568 Analysis of phagosomal proteomes: From latex-bead to bacterial phagosomes
Li, Q., Jagannath, C., Rao, P.K., Singh, C.R. and Lostumba, G.
Proteomics, ***10(22)****, 4098-4116 (2010)*

Phagosomal proteome characterization has contributed significantly to the understanding of host–pathogen interaction and the mechanism of infectious diseases caused by intracellular bacteria. The latex bead-containing phagosome has been widely used as a model system to study phagosomal proteomes at a global level. In contrast, the study of bacteria-containing phagosomes at a similar level has just begun. A number of intracellular microbial species are studied for their proteomes during the invasion of a host, providing insight into their metabolic adaptation in host cells and interaction with host-cell antimicrobial

environments. In this review, we attempt to summarize the most recent advancements in the proteomic study of microbial phagosomes, especially those originating from mouse or human cells. We also briefly describe the proteomics of latex bead-containing phagosomes because they are often used as model phagosomes for study. We provide descriptions on major biological and technological components in phagosomal proteome studies. We also discuss the role of phagosomal proteome study in the broader horizon of systems biology and the technological challenges in phagosomal proteome characterization.

3.1569 Localization and Activation of the Drosophila Protease Easter Require the ER-Resident Saposin-like Protein Seele

Stein, D., Charatsi, I., Cho, Y.S., Zhang, Z., Nguyen, J., DeLotto, R., Luschnig, S. and Moussian, B.
Current Biol., ***20(21)***, *1953-1958 (2010)*

Drosophila embryonic dorsal-ventral polarity is generated by a series of serine protease processing events in the egg perivitelline space. Gastrulation Defective processes Snake, which then cleaves Easter, which then processes Spätzle into the activating ligand for the Toll receptor [1,2,3]. *seele* was identified in a screen for mutations that, when homozygous in ovarian germline clones, lead to the formation of progeny embryos with altered embryonic patterning; maternal loss of *seele* function leads to the production of moderately dorsalized embryos [4]. By combining constitutively active versions of Gastrulation Defective, Snake, Easter, and Spätzle with loss-of-function alleles of *seele*, we find that Seele activity is dispensable for Spätzle-mediated activation of Toll but is required for Easter, Snake, and Gastrulation Defective to exert their effects on dorsal-ventral patterning. Moreover, Seele function is required specifically for secretion of Easter from the developing embryo into the perivitelline space and for Easter processing. Seele protein resides in the endoplasmic reticulum of blastoderm embryos, suggesting a role in the trafficking of Easter to the perivitelline space, prerequisite to its processing and function. Easter transport to the perivitelline space represents a previously unappreciated control point in the signal transduction pathway that controls *Drosophila* embryonic dorsal-ventral polarity.

3.1570 Stimulation of apical Cl^-/HCO_3^- (OH^-) exchanger, SLC26A3 by neuropeptide Y is lipid raft dependent

Saksena, S., Tyagi, S., Goyal, S., Gill, R.K., Alrefai, W.A., Ramaswamy, A.K. and Dudeja, P.K.
Am. J. Physiol. Gastrintest. Liver Physiol., ***299***, *G1334-G1343 (2010)*

Neuropeptide Y (NPY), an important proabsorptive hormone of the gastrointestinal tract has been shown to inhibit chloride secretion and stimulate NaCl absorption. However, mechanisms underlying the proabsorptive effects of NPY are not fully understood. The present studies were designed to examine the direct effects of NPY on apical Cl^-/HCO_3^-(OH^-) exchange activity and the underlying mechanisms involved utilizing Caco2 cells. Our results showed that NPY (100 nM, 30 min) significantly increased Cl^-/HCO_3^-(OH^-) exchange activity (~ 2-fold). Selective NPY/Y1 or Y2 receptor agonists mimicked the effects of NPY. NPY-mediated stimulation of Cl^-/HCO_3^-(OH^-) exchange activity involved the ERK1/2 MAP kinase-dependent pathway. Cell surface biotinylation studies showed that NPY does not alter DRA (apical Cl^-/HCO_3^-(OH^-) exchanger) surface expression, ruling out the involvement of membrane trafficking events. Interestingly, DRA was found to be predominantly expressed in the detergent-insoluble (DI) and low-density fractions (LDF) of human colonic apical membrane vesicles (AMVs) representing lipid rafts. Depletion of membrane cholesterol by methyl-β-cyclodextrin (MβCD, 10 mM, 1 h) remarkably decreased DRA expression in the DI fractions. Similar results were obtained in Triton-X 100-treated Caco2 plasma membranes. DRA association with lipid rafts in the DI and LDF fractions of Caco2 cells was significantly enhanced (~ 45%) by NPY compared with control. MβCD significantly decreased Cl^-/HCO_3^-(OH^-) exchange activity in Caco2 cells as measured by DIDS- or niflumic acid-sensitive $^{36}Cl^-$ uptake (~ 50%). Our results demonstrate that NPY modulates Cl^-/HCO_3^-(OH^-) exchange activity by enhancing the association of DRA with lipid rafts, thereby resulting in an increase in Cl^-/HCO_3^-(OH^-) exchange activity. Our findings suggest that the alteration in the association of DRA with lipid rafts may contribute to the proabsorptive effects of NPY in the human intestine.

3.1571 Immunospecific Responses to Bacterial Elongation Factor Tu during *Burkholderia* Infection and Immunization

Nieves, W., Heang, J., Asakrah, S., Höner zu Bentrup, K., Roy, C.J. and Morici, L.A.
PloSOne, ***5(12)***, *e14361 (2010)*

Burkholderia pseudomallei is the etiological agent of melioidosis, a disease endemic in parts of Southeast Asia and Northern Australia. Currently there is no licensed vaccine against infection with this biological threat agent. In this study, we employed an immunoproteomic approach and identified bacterial Elongation factor-Tu (EF-Tu) as a potential vaccine antigen. EF-Tu is membrane-associated, secreted in outer membrane vesicles (OMVs), and immunogenic during *Burkholderia* infection in the murine model of melioidosis. Active immunization with EF-Tu induced antigen-specific antibody and cell-mediated immune responses in mice. Mucosal immunization with EF-Tu also reduced lung bacterial loads in mice challenged with aerosolized *B. thailandensis*. Our data support the utility of EF-Tu as a novel vaccine immunogen against bacterial infection.

3.1572 The Role of Glutamate Release on Voltage-Dependent Anion Channels (VDAC)-Mediated Apoptosis in an Eleven Vessel Occlusion Model in Rats
Park, E., Lee, G-J., Choi, S.., Choi, S-K., Chae, S-J., Kang, S-W., Pak, Y.K. and Park, H-K.
PloSOne, ***5(12)***, *e15192 (2010)*

Voltage-dependent anion channel (VDAC) is the main protein in mitochondria-mediated apoptosis, and the modulation of VDAC may be induced by the excessive release of extracellular glutamate. This study examined the role of glutamate release on VDAC-mediated apoptosis in an eleven vessel occlusion model in rats. Male Sprague-Dawley rats (250–350 g) were used for the 11 vessel occlusion ischemic model, which were induced for a 10-min transient occlusion. During the ischemic and initial reperfusion episode, the real-time monitoring of the extracellular glutamate concentration was measured using an amperometric microdialysis biosensor and the cerebral blood flow (CBF) was monitored by laser-Doppler flowmetry. To confirm neuronal apoptosis, the brains were removed 72 h after ischemia to detect the neuron-specific nuclear protein and pro-apoptotic proteins (cleaved caspase-3, VDAC, p53 and BAX). The changes in the mitochondrial morphology were measured by atomic force microscopy. A decrease in the % of CBF was observed, and an increase in glutamate release was detected after the onset of ischemia, which continued to increase during the ischemic period. A significantly higher level of glutamate release was observed in the ischemia group. The increased glutamate levels in the ischemia group resulted in the activation of VDAC and pro-apoptotic proteins in the hippocampus with morphological alterations to the mitochondria. This study suggests that an increase in glutamate release promotes VDAC-mediated apoptosis in an 11 vessel occlusion ischemic model.

3.1573 Calcium Ionophore-Induced Tissue Factor (TF) Decryption Induces TF Immobilization Into Lipid Rafts and Negative Regulation of TF Procoagulant Activity
Popescu, N.I., Lupu, C. and Lupu, F.
Blood, ***116***, *Abstract 1131 (2010)*

Cell exposed tissue factor (TF), the physiologic initiator of blood coagulation, is normally expressed in a low procoagulant, or cryptic conformation, and requires activation, or decryption, to fully exhibit its procoagulant potential. TF decryption is not fully understood and multiple decrypting mechanisms have been proposed including phosphatidylserine (PS) exposure, TF monomerization, association with lipid rafts and redox modulation of TF. Calcium ionophores have been extensively used as TF decrypting agents, and both PS-dependent and independent mechanisms have been associated with ionophore-induced TF decryption. In the present study we analyzed the changes that occur in the lateral mobility of cell exposed TF during calcium ionophore-induced decryption, using a TF chimera with monomeric yellow fluorescent protein (YFP-TF). The YFP-TF expressed by endothelial cells (EC) retains TF procoagulant activity, is mainly exposed on the cell surface and can be decrypted similarly with endogenous TF by the calcium ionophore ionomycin. We analyzed the changes in TF membrane mobility during decryption using live cell imaging of YFP-TF expressed in EC. Fluorescence recovery after photobleaching (FRAP) analysis revealed a decreased mobility of TF in EC treated with the decrypting agent ionomycin. The YFP-TF fluorescence in the region of interest was more easily bleached in ionomycin–treated cells as compared with controls. The observed maximum recovery (R_{max}) of YFP-TF fluorescence in the bleached region of interest was significantly higher in control cells (80.84% recovery) as compared with ionomycin treated EC (39.29% recovery). These correlated with a decrease in YFP-TF mobile fraction from 50% for the control cells to 18% for the ionomycin treated EC. The lateral diffusion of the YFP-TF mobile fraction was similar between the two conditions, with halftime of fluorescence recovery of 7.69 sec in ionophore-treated cells and 10.69 sec in controls. These results suggest an immobilization of YFP-TF during decryption, which can be achieved by either lipid raft translocation or cytoskeleton floating. Similar to previous observations where TF cytoplasmic domain did not influence TF decryption, deletion of the TF cytoplasmic domain did not affect the lateral mobility of YFP-TF in FRAP analysis. To analyze decryption-induced changes in TF

association with lipid domains, membrane fractions were isolated on a discontinuous **Opti-Prep** density gradient. Ionomycin treatment induced YFP-TF translocation from higher density, non-raft membrane fractions toward higher-buoyancy, raft fractions. Furthermore, the observed TF translocation into lipid rafts occurs without the formation of the quaternary complex with coagulation factors FVIIa, FXa and tissue factor pathway inhibitor (TFPI), as previously described. To address the functional modulation of TF procoagulant potential in response to lipid raft translocation, cell membrane cholesterol was either depleted with methyl-β-cyclodextrine (MβCD) or supplemented from an aqueous mixture of cholesterol-MβCD. Membrane cholesterol depletion decrypted TF in EC, likely through PS exposure, while also enhancing the procoagulant potential of ionomycin-decrypted TF. In contrast, cholesterol supplementation decreases the procoagulant potential of ionomycin-decrypted TF. Taken together, these observations support the model of tonic inhibition of TF procoagulant activity by the lipid raft environment. In conclusion, by live cell imaging we show that TF membrane mobility changes during calcium-ionophore induced decryption resulting in an immobilization of TF in lipid rafts. The immobilization is not influenced by the cytoplasmic domain of TF and does not require the formation of the TF-FVIIa-FXa-TFPI quaternary complex. Translocation into lipid rafts provides tonic inhibition of TF procoagulant potential and, as a consequence, we show for the first time that decrypting agents can also initiate negative regulation of TF procoagulant function. This negative feedback loop may help convert the decrypted TF back to its cryptic, low coagulant form.

3.1574 **Naturally Produced Outer Membrane Vesicles from Pseudomonas aeruginosa Elicit a Potent Innate Immune Response via Combined Sensing of Both Lipopolysaccharide and Protein Components**
Ellis, T.N., Leiman, S.A. and Kuehn, M.J.
Infect. Immun., ***78(9)***, *3822-3831 (2010)*

Pseudomonas aeruginosa is a prevalent opportunistic human pathogen that, like other Gram-negative pathogens, secretes outer membrane vesicles. Vesicles are complex entities composed of a subset of envelope lipid and protein components that have been observed to interact with and be internalized by host cells. This study characterized the inflammatory responses to naturally produced P. aeruginosa vesicles and determined the contribution of vesicle Toll-like receptor (TLR) ligands and vesicle proteins to that response. Analysis of macrophage responses to purified vesicles by real-time PCR and enzyme-linked immunosorbent assay identified proinflammatory cytokines upregulated by vesicles. Intact vesicles were shown to elicit a profoundly greater inflammatory response than the response to purified lipopolysaccharide (LPS). Both TLR ligands LPS and flagellin contributed to specific vesicle cytokine responses, whereas the CpG DNA content of vesicles did not. Neutralization of LPS sensing demonstrated that macrophage responses to the protein composition of vesicles required the adjuvantlike activity of LPS to elicit strain specific responses. Protease treatment to remove proteins from the vesicle surface resulted in decreased interleukin-6 and tumor necrosis factor alpha production, indicating that the production of these specific cytokines may be linked to macrophage recognition of vesicle proteins. Confocal microscopy of vesicle uptake by macrophages revealed that vesicle LPS allows for binding to macrophage surfaces, whereas vesicle protein content is required for internalization. These data demonstrate that macrophage sensing of both LPS and protein components of outer membrane vesicles combine to produce a bacterial strain-specific response that is distinct from those triggered by individual, purified vesicle components.

3.1575 **Role of Lysyl Oxidase Propeptide in Secretion and Enzyme Activity**
Grimsby, J.L., Lucero, H.A., Trackman, P.C., Ravid, K. and Kagan, H.M.
J. Cell. Biochem., ***111***, *1231-1243 (2010)*

Lysyl oxidase (LOX) is secreted as a proenzyme (proLOX) that is proteolytically processed in the extracellular milieu to release the propeptide and mature, active LOX. LOX oxidizes lysyl residues of a number of protein substrates in the extracellular matrix and on the cell surface, which impacts several physiological and disease states. Although the LOX propeptide (LOX-PP) is glycosylated, little is known about the role of this modification in LOX secretion and activity. To gain insight into this issue, cells were transfected with native, full-length LOX cDNA (pre-pro-LOX), the N-glycosylation null pre-[N/Q]pro-LOX cDNA and the deletion mutant pre-LOX cDNA, referred to as secretory LOX, in which mature LOX is targeted to the secretory pathway without its N-terminal propeptide sequence. The results show that glycosylation of the LOX-PP is not required for secretion and extracellular processing of pro-LOX but it is required for optimal enzyme activity of the resulting mature LOX. Complete deletion of the propeptide sequence prevents mature LOX from exiting the endoplasmic reticulum (ER). Taken together, our study points out the requirement of the LOX-PP for pro-LOX exit from the ER and is the first to highlight the influence of LOX-PP glycosylation on LOX enzyme activity.

3.1576 **Improved sub-cellular resolution *via* simultaneous analysis of organelle proteomics data across varied experimental conditions**
Trotter, M.W.B., Sadowski, P.G., Dunkley, T.P.J., Groen, A.J. and Lilley, K.S.
Proteomics, ***10(23)***, *4213-4219 (2010)*

Spatial organisation of proteins according to their function plays an important role in the specificity of their molecular interactions. Emerging proteomics methods seek to assign proteins to sub-cellular locations by partial separation of organelles and computational analysis of protein abundance distributions among partially separated fractions. Such methods permit simultaneous analysis of unpurified organelles and promise proteome-wide localisation in scenarios wherein perturbation may prompt dynamic re-distribution. Resolving organelles that display similar behavior during a protocol designed to provide partial enrichment represents a possible shortcoming. We employ the Localisation of Organelle Proteins by Isotope Tagging (LOPIT) organelle proteomics platform to demonstrate that combining information from distinct separations of the same material can improve organelle resolution and assignment of proteins to sub-cellular locations. Two previously published experiments, whose distinct gradients are alone unable to fully resolve six known protein–organelle groupings, are subjected to a rigorous analysis to assess protein–organelle association *via* a contemporary pattern recognition algorithm. Upon straightforward combination of single-gradient data, we observe significant improvement in protein–organelle association *via* both a non-linear support vector machine algorithm and partial least-squares discriminant analysis. The outcome yields suggestions for further improvements to present organelle proteomics platforms, and a robust analytical methodology *via* which to associate proteins with sub-cellular organelles.

3.1577 **Oxysterol Binding Protein-dependent Activation of Sphingomyelin Synthesis in the Golgi Apparatus Requires Phosphatidylinositol 4-Kinase IIα**
Banerji, S., Ngo, M., Lane, C.F., Robinson, C-A., Minogue, S. and Ridgway, N.D.
Mol. Biol. Cell, ***21***, *4141-4150 (2010)*

Cholesterol and sphingomyelin (SM) associate in raft domains and are metabolically coregulated. One aspect of coordinate regulation occurs in the Golgi apparatus where oxysterol binding protein (OSBP) mediates sterol-dependent activation of ceramide transport protein (CERT) activity and SM synthesis. Because CERT transfer activity is dependent on its phosphatidylinositol 4 phosphate [PtdIns(4)P]-specific pleckstrin homology domain, we investigated whether OSBP activation of CERT involved a Golgi-associated PtdIns 4-kinase (PI4K). Cell fractionation experiments revealed that Golgi/endosome-enriched membranes from 25-hydroxycholesterol-treated Chinese hamster ovary cells had increased activity of a sterol-sensitive PI4K that was blocked by small interfering RNA silencing of OSBP. Consistent with this sterol-requirement, OSBP silencing also reduced the cholesterol content of endosome/*trans*-Golgi network (TGN) fractions containing PI4KIIα. PI4KIIα, but not PI4KIIIβ, was required for oxysterol-activation of SM synthesis and recruitment of CERT to the Golgi apparatus. However, neither PI4KIIα nor PI4KIIIβ expression was required for 25-hydroxycholesterol–dependent translocation of OSBP to the Golgi apparatus. The presence of OSBP, CERT, and PI4KIIα in the TGN of oxysterol-stimulated cells suggests that OSBP couples sterol binding or transfer activity with regulation of PI4KIIα activity, leading to CERT recruitment to the TGN and increased SM synthesis.

3.1578 **Reduced levels of folate transporters (PCFT and RFC) in membrane lipid rafts result in colonic folate malabsorption in chronic alcoholism**
Wani, N.A. and Kaur, J.
Cell. Physiol., ***226***, *579-587 (2011)*

We studied the effect of chronic ethanol ingestion on folate transport across the colonic apical membranes (CAM) in rats. Male Wistar rats were fed 1 g/kg body weight/day ethanol (20%) solution orally for 3 months and folate transport was studied in the isolated colon apical membrane vesicles. The folate transport was found to be carrier mediated, saturable, with pH optima at 5.0. Chronic ethanol ingestion reduced the folate transport across the CAM by decreasing the affinity of transporters (high Km) for the substrate and by decreasing the number of transporter molecules (low Vmax) on the colon luminal surface. The decreased transport activity at the CAM was associated with down-regulation of the proton-coupled folate transporter (PCFT) and the reduced folate carrier (RFC) which resulted in decreased PCFT and RFC protein levels in the colon of rats fed alcohol chronically. Moreover, the PCFT and the RFC were found to be distributed in detergent insoluble fraction of the CAM in rats. Floatation experiments on Optiprep density gradients demonstrated the association of the PCFT and the RFC protein with lipid rafts (LR).

Chronic alcoholism decreased the PCFT and the RFC protein levels in the CAM LR in accordance with the decreased synthesis. Hence, we propose that downregulation in the expression of the PCFT and the RFC in colon results in reduced levels of these transporters in colon apical membrane LR as a mechanism of folate malabsorption during chronic alcoholism.

3.1579 The Tethering Arm of the EGF Receptor Is Required for Negative Cooperativity and Signal Transduction
Adak, S., DeAndrade, D. and Pike, L.J.
J. Biol. Chem., ***286(2)***, *1545-1555 (2011)*

The EGF receptor is a classical receptor-tyrosine kinase. In the absence of ligand, the receptor adopts a closed conformation in which the dimerization arm of subdomain II interacts with the tethering arm in subdomain IV. Following the binding of EGF, the receptor opens to form a symmetric, back-to-back dimer. Although it is clear that the dimerization arm of subdomain II is central to the formation of receptor dimers, the role of the tethering arm of subdomain IV (residues 561–585) in this configuration is not known. Here we use ^{125}I-EGF binding studies to assess the functional role of the tethering arm in the EGF receptor dimer. Mutation of the three major residues that contribute to tethering (D563A,H566A,K585A-EGF receptor) did not significantly alter either the ligand binding properties or the signaling properties of the EGF receptor. By contrast, breaking the Cys^{558}-Cys^{567} disulfide bond through double alanine replacements or deleting the loop entirely led to a decrease in the negative cooperativity in EGF binding and was associated with small changes in downstream signaling. Deletion of the Cys^{571}-Cys^{593} disulfide bond abrogated cooperativity, resulting in a high affinity receptor and increased sensitivity of downstream signaling pathways to EGF. Releasing the Cys^{571}-Cys^{593} disulfide bond resulted in extreme negative cooperativity, ligand-independent kinase activity, and impaired downstream signaling. These data demonstrate that the tethering arm plays an important role in supporting cooperativity in ligand binding. Because cooperativity implies subunit-subunit interactions, these results also suggest that the tethering arm contributes to intersubunit interactions within the EGF receptor dimer.

3.1580 Differential roles for SUR subunits in KATP channel membrane targeting and regulation
Hund, T.J. and Mohler, P.J.
Am. J. Physiol. Heart Circ. Physiol., ***300***, *H33-H35 (2011)*

No abstracts available.

3.1581 Native IL-32 is released from intestinal epithelial cells via a non-classical secretory pathway as a membrane-associated protein
Hasegawa, H., Thomas, H.J., Schooley, K. and Born, T.L.
Cytokine, ***53***, *74-83 (2011)*

Although IL-32 has been shown to be induced under various pathological conditions, a detailed understanding of native IL-32 intracellular distribution and mechanism of release from cells has not been reported. We examined the expression of IL-32 in the intestinal epithelial cell line HT-29 following TNFα and IFNγ co-stimulation. The subcellular localization of induced IL-32 was associated with the membrane of lipid droplet-like structures and vacuolar structures that co-localized with markers of endosomes and lysosomes. Prolonged co-stimulation resulted in cell death and appearance of IL-32 in the culture medium. IL-32 released from co-stimulated HT-29 cells was found in a detergent-sensitive particulate fraction, and in a step density gradient the IL-32 particulate was buoyant, suggesting association with a membrane-bound vesicle. Upon Triton X-114 partitioning, most of the IL-32 partitioned to the detergent phase, suggesting hydrophobic characteristics. When IL-32-containing vesicles were subjected to protease K treatment, a protease resistant ~12 kDa fragment was generated from ~24 kDa IL-32. We propose that under these conditions, native IL-32 is released via a non-classical secretory route perhaps involving multi-vesicular bodies and exosomes. Demonstration of membrane association for both intracellular and released IL-32 suggests this unique cytokine may have a complex biosynthetic pathway and mechanism of action.

3.1582 Reconfiguring polylysine architectures for controlling polyplex binding and non-viral transfection
Parelkar, S.S., Chan-Seng, D. and Emrick, T.
Biomaterials, ***32***, *2432-2444 (2011)*

Poly(L-lysine) (PLL) is a cationic polyelectrolyte of interest for many applications, including in therapeutic biology for DNA complexation and transfection. Several non-lysine based polycations have been shown to

afford more efficient transfection in live cells than has been achieved with PLL. We find that reconfiguring polylysine into short oligolysine grafts, strung from a hydrophobic polymer backbone, gives transfection reagents greatly superior to PLL, despite having the identical cationic functional groups (*i.e.*, exclusively primary amines). Altering the oligolysine graft length modulates DNA-polymer interactions and transfection efficiency, while incorporating the PKKKRKV heptapeptide (the Simian virus SV40 large T-antigen nuclear localization sequence) pendent groups onto the polymer backbone led to even greater transfection efficiency over the oligolysine-grafted structures. Protein expression levels obtained with these novel polymer transfection reagents were higher than, or comparable to, expression seen in the cases of JetPEI™, FuGENE® 6 and Lipofectamine™ 2000, the later being notorious for cytotoxicity that accompanies high transfection efficiency. The relative strength of the polymer-DNA complex is key to the transfection performance, as judged by serum stability and PicoGreen analysis. Moreover, polyplexes formed from our graft copolymer structures exhibit low cytotoxicity, contributing to the therapeutic promise of these novel reagents.

3.1583 Purification and characterization of HIV–human protein complexes
Jäger, S., Gulbache, N., Cimermancic, P., Kane, J., He, N., Chou, S., D'Orso, I., Fernandes, J., Jang, G., Frankel, A.D., Alber, T. and Zhou, Q.
*Methods, **53**, 13-19 (2011)*

To fully understand how pathogens infect their host and hijack key biological processes, systematic mapping of intra-pathogenic and pathogen–host protein–protein interactions (PPIs) is crucial. Due to the relatively small size of viral genomes (usually around 10–100 proteins), generation of comprehensive host–virus PPI maps using different experimental platforms, including affinity tag purification-mass spectrometry (AP-MS) and yeast two-hybrid (Y2H) approaches, can be achieved. Global maps such as these provide unbiased insight into the molecular mechanisms of viral entry, replication and assembly. However, to date, only two-hybrid methodology has been used in a systematic fashion to characterize viral–host protein–protein interactions, although a deluge of data exists in databases that manually curate from the literature individual host–pathogen PPIs. We will summarize this work and also describe an AP-MS platform that can be used to characterize viral-human protein complexes and discuss its application for the HIV genome.

3.1584 Immunization with Salmonella enterica Serovar Typhimurium-Derived Outer Membrane Vesicles Delivering the Pneumococcal Protein PspA Confers Protection against Challenge with Streptococcus pneumoniae
Muralinath, M., Kuehn, M.J., Roland, K.L. and Curtiss III, R.
*Infect. Immun., **79(2)**, 887-894 (2011)*

Gram-negative bacteria produce outer membrane vesicles (OMVs) that serve a variety of functions related to survival and pathogenicity. Periplasmic and outer membrane proteins are naturally captured during vesicle formation. This property has been exploited as a method to derive immunogenic vesicle preparations for use as vaccines. In this work, we constructed a Salmonella enterica serovar Typhimurium strain that synthesized a derivative of the pneumococcal protein PspA engineered to be secreted into the periplasmic space. Vesicles isolated from this strain contained PspA in the lumen. Mice intranasally immunized with the vesicle preparation developed serum antibody responses against vesicle components that included PspA and Salmonella-derived lipopolysaccharide and outer membrane proteins, while no detectable responses developed in mice immunized with an equivalent dose of purified PspA. Mucosal IgA responses developed against the Salmonella components, while the response to PspA was less apparent in most mice. Mice immunized with the vesicle preparation were completely protected against a 10x 50% lethal dose (LD50) challenge of Streptococcus pneumoniae and significantly protected against a 200x LD50 challenge, while control mice immunized with purified PspA or empty vesicles were not protected. These results establish that vesicles can be used to mucosally deliver an antigen from a Gram-positive organism and induce a protective immune response.

3.1585 αB-Crystallin Is Found in Detergent-resistant Membrane Microdomains and Is Secreted via Exosomes from Human Retinal Pigment Epithelial
Gangalum, R., Atanasov, I.C., Zhou,, Z.H. and Bhat, S.P.
*J. Biol. Chem., **286(5)**, 3261-3269 (2011)*

αB-crystallin (αB) is known as an intracellular Golgi membrane-associated small heat shock protein. Elevated levels of this protein have been linked with a myriad of neurodegenerative pathologies including

Alzheimer disease, multiple sclerosis, and age-related macular degeneration. The membrane association of αB has been known for more than 3 decades, yet its physiological import has remained unexplained. In this investigation we show that αB is secreted from human adult retinal pigment epithelial cells via microvesicles (exosomes), independent of the endoplasmic reticulum-Golgi protein export pathway. The presence of αB in these lipoprotein structures was confirmed by its susceptibility to digestion by proteinase K only when exosomes were exposed to Triton X-100. Transmission electron microscopy was used to localize αB in immunogold-labeled intact and permeabilized microvesicles. The saucer-shaped exosomes, with a median diameter of 100–200 nm, were characterized by the presence of flotillin-1, α-enolase, and Hsp70, the same proteins that associate with detergent-resistant membrane microdomains (DRMs), which are known to be involved in their biogenesis. Notably, using polarized adult retinal pigment epithelial cells, we show that the secretion of αB is predominantly apical. Using OptiPrep gradients we demonstrate that αB resides in the DRM fraction. The secretion of αB is inhibited by the cholesterol-depleting drug, methyl β-cyclodextrin, suggesting that the physiological function of this protein and the regulation of its export through exosomes may reside in its association with DRMs/lipid rafts.

3.1586 FcγRIIIb Triggers Raft-dependent Calcium Influx in IgG-mediated Responses in Human Neutrophils

Marois, L., Pare, G., Vaillancourt, M., Rolelt-labelle, E. and Naccache, P.H.
J. Biol. Chem., ***286(5)***, *3509-3519 (2011)*

Human neutrophils constitutively express a unique combination of FcγRs, namely FcγRIIa and FcγRIIIb. Numerous lines of evidence support the concept that these FcγRs generate only partially characterized intracellular signals. However, despite the fact that both receptors are likely to be engaged simultaneously in a physiological setting, no recent publications have investigated the distinct, although partially convergent, results of their joint activation in IgG-dependent responses. To examine the significance of the co-expression of FcγRIIa and FcγRIIIb on human neutrophils, we analyzed the neutrophil responses to stimuli that engage these FcγRs, namely the phagocytosis of human IgG-opsonized zymosan and the responses to heat-aggregated IgGs. Blocking antibodies to either FcγR significantly decreased the phagocytic index and the stimulated production of superoxide anions. Both receptors are required for optimal IgG-dependent responses by human neutrophils. On the other hand, only blocking antibodies to FcγRIIIb, but not to FcγRIIa, inhibited the mobilization of calcium in response to heat-aggregated IgGs. Furthermore, phagocytosis of IgG-opsonized zymosan by human neutrophils required an extracellular influx of calcium that was blocked only by antibodies against FcγRIIIb. We also observed that this calcium influx as well as the IgG-dependent phagocytosis were dependent on the integrity of the plasma membrane detergent-resistant microdomains to which both isoforms were recruited following stimulation by heat-aggregated IgGs. These data clarify the mechanisms that regulate the FcγRs constitutively expressed on human neutrophils, describe a specific contribution of FcγRIIIb at the level of the mobilization of calcium, and provide evidence for a crucial role of detergent-resistant microdomains in this process.

3.1587 Caspase-8 and caspase-7 sequentially mediate proteolytic activation of acid sphingomyelinase in TNF-R1 receptosomes

Edelmann, B., Bertsch, U., Tchikov, V., Winoto-Morbach, S., Perrotta, C., Jakob, M., Adam-Klages, S., Kabelitz, D. and Schütze, S.
EMBO J., ***30(2)***, *379-394 (2011)*

We previously demonstrated that tumour necrosis factor (TNF)-induced ceramide production by endosomal acid sphingomyelinase (A-SMase) couples to apoptosis signalling via activation of cathepsin D and cleavage of Bid, resulting in caspase-9 and caspase-3 activation. The mechanism of TNF-mediated A-SMase activation within the endolysosomal compartment is poorly defined. Here, we show that TNF-induced A-SMase activation depends on functional caspase-8 and caspase-7 expression. The active forms of all three enzymes, caspase-8, caspase-7 and A-SMase, but not caspase-3, colocalize in internalized TNF receptosomes. While caspase-8 and caspase-3 are unable to induce activation of purified pro-A-SMase, we found that caspase-7 mediates A-SMase activation by direct interaction resulting in proteolytic cleavage of the 72-kDa pro-A-SMase zymogen at the non-canonical cleavage site after aspartate 253, generating an active 57 kDa A-SMase molecule. Caspase-7 down modulation revealed the functional link between caspase-7 and A-SMase, confirming proteolytic cleavage as one further mode of A-SMase activation. Our data suggest a signalling cascade within TNF receptosomes involving sequential activation of caspase-8 and caspase-7 for induction of A-SMase activation by proteolytic cleavage of pro-A-SMase.

3.1588 **The phosphoinositide 3-kinase Vps34p is required for pexophagy in *Saccharomyces cerevisiae***
Grunau, S., Lay, D., Mindthoff, S., Platta, H.W., Girzalsky, W., Just, W.W. and Erdmann, R.
Biochem. J., ***434***, *161-170 (2011)*

PIds (phosphoinositides) are phosphorylated derivatives of the membrane phospholipid PtdIns that have emerged as key regulators of many aspects of cellular physiology. We have discovered a PtdIns3*P*-synthesizing activity in peroxisomes of *Saccharomyces cerevisiae* and have demonstrated that the lipid kinase Vps34p is already associated with peroxisomes during biogenesis. However, although Vps34 is required, it is not essential for optimal peroxisome biogenesis. The function of Vps34p-containing complex I as well as a subset of PtdIns3*P*-binding proteins proved to be mandatory for the regulated degradation of peroxisomes. This demonstrates that PtdIns3*P*-mediated signalling is required for pexophagy.

3.1589 **An RNA-zipcode-independent mechanism that localizes Dia1 mRNA to the perinuclear ER through interactions between Dia1 nascent peptide and Rho–GTP**
Liao, G., ma, X. and Liu, G.
J. Cell Sci., ***124***, *589-599 (2011)*

Signal-peptide-mediated ER localization of mRNAs encoding for membrane and secreted proteins, and RNA-zipcode-mediated intracellular targeting of mRNAs encoding for cytosolic proteins are two well-known mechanisms for mRNA localization. Here, we report a previously unidentified mechanism by which mRNA encoding for Dia1, a cytosolic protein without the signal peptide, is localized to the perinuclear ER in an RNA-zipcode-independent manner in fibroblasts. *Dia1* mRNA localization is also independent of the actin and microtubule cytoskeleton but requires translation and the association of Dia1 nascent peptide with the ribosome–mRNA complex. Sequence mapping suggests that interactions of the GTPase binding domain of Dia1 peptide with active Rho are important for *Dia1* mRNA localization. This mechanism can override the β-actin RNA zipcode and redirect β-actin mRNA to the perinuclear region, providing a new way to manipulate intracellular mRNA localization.

3.1590 **Sorting receptor Rer1 controls surface expression of muscle acetylcholine receptors by ER retention of unassembled α-subunits**
Valkova, C., Albrizio, M., Röder, I.V., Schwake, M., Betto, R., Rudolf, R. and Kaether, C.
PNAS, ***108(2)***, *621-625 (2011)*

The nicotinic acetylcholine receptor of skeletal muscle is composed of five subunits that are assembled in a stepwise manner. Quality control mechanisms ensure that only fully assembled receptors reach the cell surface. Here, we show that Rer1, a putative Golgi-ER retrieval receptor, is involved in the biogenesis of acetylcholine receptors. Rer1 is expressed in the early secretory pathway in the myoblast line C2C12 and in mouse skeletal muscle, and up-regulated during myogenesis. Upon down-regulation of Rer1 in C2C12 cells, unassembled acetylcholine receptor α-subunits escape from the ER and are transported to the plasma membrane and lysosomes, where they are degraded. As a result, the amount of fully assembled receptor at the cell surface is reduced. In vivo Rer1 knockdown and genetic inactivation of one Rer1 allele lead to significantly smaller neuromuscular junctions in mice. Our data show that Rer1 is a functionally important unique factor that controls surface expression of muscle acetylcholine receptors by localizing unassembled α-subunits to the early secretory pathway.

3.1591 **A role for oxysterol-binding protein–related protein 5 in endosomal cholesterol trafficking**
Du, X., Kumar, J., Ferguson, C., Schulz, T.A., Ong, Y.S., Hong, W., Prinz, W.A., Parton, R.G., Brown, A.J. and Yang, H.
J. Cell Biol., ***192(1)***, *121-135 (2011)*

Oxysterol-binding protein (OSBP) and its related proteins (ORPs) constitute a large and evolutionarily conserved family of lipid-binding proteins that target organelle membranes to mediate sterol signaling and/or transport. Here we characterize ORP5, a tail-anchored ORP protein that localizes to the endoplasmic reticulum. Knocking down ORP5 causes cholesterol accumulation in late endosomes and lysosomes, which is reminiscent of the cholesterol trafficking defect in Niemann Pick C (NPC) fibroblasts. Cholesterol appears to accumulate in the limiting membranes of endosomal compartments in ORP5-depleted cells, whereas depletion of NPC1 or both ORP5 and NPC1 results in luminal accumulation of

cholesterol. Moreover, trans-Golgi resident proteins mislocalize to endosomal compartments upon ORP5 depletion, which depends on a functional NPC1. Our results establish the first link between NPC1 and a cytoplasmic sterol carrier, and suggest that ORP5 may cooperate with NPC1 to mediate the exit of cholesterol from endosomes/lysosomes.

3.1592 Regulation of vascular endothelial growth factor receptor 2 trafficking and angiogenesis by Golgi localized t-SNARE syntaxin 6

Manickam, V., Tiwari, A., Jung, J-J., Bhattacharya, R., Goel, A., Mukhopadhyay, D. and Choudhury, A.
*Blood, **117(4)**, 1425-1435 (2011)*

Vascular endothelial growth factor receptor 2 (VEGFR2) plays a key role in physiologic and pathologic angiogenesis. Plasma membrane (PM) levels of VEGFR2 are regulated by endocytosis and secretory transport through the Golgi apparatus. To date, the mechanism whereby the VEGFR2 traffics through the Golgi apparatus remains incompletely characterized. We show in human endothelial cells that binding of VEGF to the cell surface localized VEGFR2 stimulates exit of intracellular VEGFR2 from the Golgi apparatus. Brefeldin A treatment reduced the level of surface VEGFR2, confirming that VEGFR2 traffics through the Golgi apparatus en route to the PM. Mechanistically, we show that inhibition of syntaxin 6, a Golgi-localized target membrane-soluble N-ethylmaleimide attachment protein receptor (t-SNARE) protein, interferes with VEGFR2 trafficking to the PM and facilitates lysosomal degradation of the VEGFR2. In cell culture, inhibition of syntaxin 6 also reduced VEGF-induced cell proliferation, cell migration, and vascular tube formation. Furthermore, in a mouse ear model of angiogenesis, an inhibitory form of syntaxin 6 reduced VEGF-induced neovascularization and permeability. Our data demonstrate the importance of syntaxin 6 in the maintenance of cellular VEGFR2 levels, and suggest that the inhibitory form of syntaxin 6 has good potential as an antiangiogenic agent.

3.1593 Effector granules in human T lymphocytes: the luminal proteome of secretory lysosomes from human T cells

Schmidt, H., Gelhaus, C., Nebendahl, M., Lettau, M., Lucius, R., Leippe, M., Kabelitz, D. and Janssen, O.
*Cell Comm. And Singnalling, **9**, 4-18 (2011)*

Background
Cytotoxic cells of the immune system have evolved a lysosomal compartment to store and mobilize effector molecules. In T lymphocytes and NK cells, the death factor FasL is one of the characteristic marker proteins of these so-called secretory lysosomes, which combine properties of conventional lysosomes and exocytotic vesicles. Although these vesicles are crucial for immune effector function, their protein content in T cells has so far not been investigated in detail.
Results
In the present study, intact membranous vesicles were enriched from homogenates of polyclonally activated T cells and initially characterized by Western blotting and electron microscopic inspection. The vesicular fraction that contained the marker proteins of secretory lysosomes was subsequently analyzed by 2D electrophoresis and mass spectrometry. The proteome analysis and data evaluation revealed that 70% of the 397 annotated proteins had been associated with different lysosome-related organelles in previous proteome studies.
Conclusion
We provide the first comprehensive proteome map of T cell-derived secretory lysosomes with only minor contaminations by cytosolic, nuclear or other proteins. This information will be useful to more precisely address the activation-dependent maturation and the specific distribution of effector organelles and proteins in individual T or NK cell populations in future studies.

3.1594 Reverse Engineering Gene Network Identifies New Dysferlin-interacting Proteins

Cacciottolo, M., Belcastro, v., Laval, S., Bushby, K., di Bernardo, D. and Nigro, v.
*J. Biol. Chem., **286(7)**, 5404-5413 (2011)*

Dysferlin (DYSF) is a type II transmembrane protein implicated in surface membrane repair of muscle. Mutations in dysferlin lead to Limb Girdle Muscular Dystrophy 2B (LGMD2B), Miyoshi Myopathy (MM), and Distal Myopathy with Anterior Tibialis onset (DMAT). The DYSF protein complex is not well understood, and only a few protein-binding partners have been identified thus far. To increase the set of interacting protein partners for DYSF we recovered a list of predicted interacting protein through a systems biology approach. The predictions are part of a “reverse-engineered” genome-wide human gene regulatory network obtained from experimental data by computational analysis. The reverse-engineering algorithm

behind the analysis relates genes to each other based on changes in their expression patterns. DYSF and AHNAK were used to query the system and extract lists of potential interacting proteins. Among the 32 predictions the two genes share, we validated the physical interaction between DYSF protein with moesin (MSN) and polymerase I and transcript release factor (PTRF) in mouse heart lysate, thus identifying two novel Dysferlin-interacting proteins. Our strategy could be useful to clarify Dysferlin function in intracellular vesicles and its implication in muscle membrane resealing.

3.1595 Folding, Quality Control, and Secretion of Pancreatic Ribonuclease in Live Cells
Geiger, R., Gautschi, M., Thor, F., Hayer, A. and Helenius, A.
J. Biol. Chem., ***286(7)***, *5813-5822 (2011)*

Although bovine pancreatic RNase is one of the best characterized proteins in respect to structure and *in vitro* refolding, little is known about its synthesis and maturation in the endoplasmic reticulum (ER) of live cells. We expressed the RNase in live cells and analyzed its folding, quality control, and secretion using pulse-chase analysis and other cell biological techniques. In contrast to the slow *in vitro* refolding, the protein folded almost instantly after translation and translocation into the ER lumen ($t_{½}$ < 3 min). Despite high stability of the native protein, only about half of the RNase reached a secretion competent, monomeric form and was rapidly transported from the rough ER via the Golgi complex ($t_{½}$ = 16 min) to the extracellular space ($t_{½}$ = 35 min). The rest remained in the ER mainly in the form of dimers and was slowly degraded. The dimers were most likely formed by C-terminal domain swapping since mutation of Asn^{113}, a residue that stabilizes such dimers, to Ser increased the efficiency of secretion from 59 to 75%. Consistent with stringent ER quality control *in vivo*, the secreted RNase in the bovine pancreas was mainly monomeric, whereas the enzyme present in the cells also contained 20% dimers. These results suggest that the efficiency of secretion is not only determined by the stability of the native protein but by multiple factors including the stability of secretion-incompetent side products of folding. The presence of *N*-glycans had little effect on the folding and secretion process.

3.1596 Calsenilin is degraded by the ubiquitin–proteasome pathway
Jang, C., Choi, J-K., Kim, E., Park, E-S., Wasco, W., Buxbaum, J.D., Kim, Y-S. and Choi, E-K.
Biochem. Biophys. Res. Comm., ***405***, *180-185 (2011)*

Calsenilin, a neuronal calcium binding protein that has been shown to have multiple functions in the cell, interacts with presenilin 1 (PS1) and presenilin 2 (PS2), represses gene transcription and binds to A-type voltage-gated potassium channels. In addition, increased levels of calsenilin are observed in the brains of Alzheimer's disease and epilepsy patients. The present study was designed to investigate the molecular mechanism of calsenilin degradation pathways in cultured cells. Here, we demonstrate that inhibition of the ubiquitin–proteasomal pathway (UPP) but not lysosomal pathway markedly increased the expression levels of calsenilin. Immunofluorescence analysis revealed that following proteasomal inhibition calsenilin accumulated in the endoplasmic reticulum (ER) and Golgi, while lysosomal inhibition had no effect on calsenilin localization. In addition, we found the change of subcellular localization of PS1 from diffuse pattern to punctuate staining pattern in the ER and perinuclear region in the presence of calsenilin. These findings suggest that calsenilin degradation is primarily mediated by the UPP and that impairment in the UPP may contribute to the involvement of calsenilin in disease-associated neurodegeneration.

3.1597 Porcine Sialoadhesin (CD169/Siglec-1) Is an Endocytic Receptor that Allows Targeted Delivery of Toxins and Antigens to Macrophages
Delputte, P.L., Van Gorp, H., Favoreel, H.W., Hoebeke, I., Delrue, I., Dewerchin, H., Verdonck, F., Verhasselt, B., Cox, E. and Nauwynck, H.J.
PloSOne, ***6(2)***, *e16827 (2011)*

Sialoadhesin is exclusively expressed on specific subpopulations of macrophages. Since sialoadhesin-positive macrophages are involved in inflammatory autoimmune diseases, such as multiple sclerosis, and potentially in the generation of immune responses, targeted delivery of drugs, toxins or antigens via sialoadhesin-specific immunoconjugates may prove a useful therapeutic strategy. Originally, sialoadhesin was characterized as a lymphocyte adhesion molecule, though recently its involvement in internalization of sialic acid carrying pathogens was shown, suggesting that sialoadhesin is an endocytic receptor. In this report, we show that porcine sialoadhesin-specific antibodies and $F(ab')_2$ fragments trigger sialoadhesin internalization, both in primary porcine macrophages and in cells expressing recombinant porcine sialoadhesin. Using chemical inhibitors, double immunofluorescence stainings and dominant-negative constructs, porcine sialoadhesin internalization was shown to be clathrin- and Eps15-dependent and to

result in targeting to early endosomes but not lysosomes. Besides characterizing the sialoadhesin endocytosis mechanism, two sialoadhesin-specific immunoconjugates were evaluated. We observed that porcine sialoadhesin-specific immunotoxins efficiently kill sialoadhesin-expressing macrophages. Furthermore, porcine sialoadhesin-specific albumin immunoconjugates were shown to be internalized in macrophages and immunization with these immunoconjugates resulted in a rapid and robust induction of albumin-specific antibodies, this compared to immunization with albumin alone. Together, these data expand sialoadhesin functionality and show that it can function as an endocytic receptor, a feature that cannot only be misused by sialic acid carrying pathogens, but that may also be used for specific targeting of toxins or antigens to sialoadhesin-expressing macrophages.

3.1598 Glucocorticoid-Induced Fetal Programming Alters the Functional Complement of Angiotensin Receptor Subtypes Within the Kidney

Gwathmey, T.M., Shaltout, H.A., Rose, J.C., Diz, D.I. and Chappell, M.C.
Hypertension, ***57(2)***, *620-626 (2011)*

We examined the impact of fetal programming on the functional responses of renal angiotensin receptors. Fetal sheep were exposed in utero to betamethasone (BMX; 0.17 mg/kg) or control (CON) at 80 to 81 days gestation with full-term delivery. Renal nuclear and plasma membrane fractions were isolated from sheep age 1.0 to 1.5 years for receptor binding and fluorescence detection of reactive oxygen species (ROS) or nitric oxide (NO). Mean arterial blood pressure and blood pressure variability were significantly higher in the BMX-exposed adult offspring versus CON sheep. The proportion of nuclear AT_1 receptors sensitive to losartan was 2-fold higher (67±6% vs 27±9%; $P<0.01$) in BMX compared with CON. In contrast, the proportion of AT_2 sites was only one third that of controls (BMX, 25±11% vs CON, 78±4%; $P<0.01$), with a similar reduction in sites sensitive to the Ang-(1-7) antagonist D-Ala7-Ang-(1-7) with BMX exposure. Functional studies revealed that Ang II stimulated ROS to a greater extent in BMX than in CON sheep (16±3% vs 6±4%; $P<0.05$); however, NO production to Ang II was attenuated in BMX (26±7% vs 82±14%; $P<0.05$). BMX exposure was also associated with a reduction in the Ang-(1-7) NO response (75±8% vs 131±26%; $P<0.05$). We conclude that altered expression of angiotensin receptor subtypes may be one mechanism whereby functional changes in NO- and ROS-dependent signaling pathways may favor the sustained increase in blood pressure evident in fetal programming.

3.1599 Multidrug Resistance-Related Protein 1 (MRP1) Function and Localization Depend on Cortical Actin

Hummel, I., Klappe, K., Ercan, C. and Kok, J.W.
Mol. Pharmacol., ***79(2)***, *229-240 (2011)*

MRP1 (ABCC1) is known to be localized in lipid rafts. Here we show in two different cell lines that localization of Mrp1/MRP1 (Abcc1/ABCC1) in lipid rafts and its function as an efflux pump are dependent on cortical actin. Latrunculin B disrupts both cortical actin and actin stress fibers. This results in partial loss of actin and Mrp1/MRP1 (Abcc1/ABCC1) from detergent-free lipid raft fractions, partial internalization of Mrp1/MRP1 (Abcc1/ABCC1), and reduction of Mrp1/MRP1 (Abcc1/ABCC1)-mediated efflux. Pretreatment with nocodazole prevents latrunculin B-induced loss of cortical actin and all effects of latrunculin B on Mrp1 (Abcc1) localization and activity. However, pretreatment with tyrphostin A23 does not prevent latrunculin B-induced loss of cortical actin, lipid raft association, and efflux activity, but it does prevent latrunculin B-induced internalization of Mrp1 (Abcc1). Cytochalasin D disrupts actin stress fibers but not cortical actin and this inhibitor much less affects Mrp1/MRP1 (Abcc1/ABCC1) localization in lipid rafts, internalization, and efflux activity. In conclusion, cortical actin disruption results in reduced Mrp1/MRP1 (Abcc1/ABCC1) activity concomitant with a partial shift of Mrp1/MRP1 (Abcc1/ABCC1) out of lipid raft fractions and partial internalization of the ABC transporter. The results suggest that reduced Mrp1 (Abcc1) function is correlated to the loss of lipid raft association but not internalization of Mrp1 (Abcc1).

3.1600 Advances in membranous vesicle and exosome proteomics improving biological understanding and biomarker discovery

Raimondo, F., Morosi, L., Chinello, C., Magni, F. and Pitto, M.
Proteomics, ***11(4)***, *709-720 (2011)*

Exosomes are membranous vesicles released by cells in extracellular fluids: they have been found and analyzed in blood, urine, amniotic fluid, breast milk, seminal fluid, saliva and malignant effusions, besides conditioned media from different cell lines. Several recent papers show that exosome proteomes of

different origin include both a common set of membrane and cytosolic proteins, and specific subsets of proteins, likely correlated to cell-type associated functions. This is particularly interesting in relation to their possible involvement in human diseases. The knowledge of exosome proteomics can help not only in understanding their biological roles but also in supplying new biomarkers to be searched for in patients' fluids. This review offers an overview of technical and analytical issues in exosome proteomics, and it highlights the significance of proteomic studies in terms of biological and clinical usefulness.

3.1601 **Recent advances in the use of *Sus scrofa* (pig) as a model system for proteomic studies**
Verma, N., Rettenmeier, A.W. and Schmitz-Spanke, S.
Proteomics, ***11(4)***, *776-793 (2011)*

Of the numerous animal models available for proteomic studies only a small number have been successfully used in understanding human biology. To date, rodents have been widely employed in proteomic and genomic studies but often these models do not truly mimic the relevant human conditions. On the other hand, the pig shows similarity in size, shape and physiology to human and has been used as a major mammalian model for many studies concerning xenotransplantation, cardiovascular diseases, blood dynamics, nutrition, general metabolic functions, digestive-related disorders, respiratory diseases, diabetes, kidney and bladder diseases, organ-specific toxicity, dermatology and neurological sequelae. With the substantially improved knowledge of the structure and function of the pig genome in the last two decades it has been found that this animal shares a high sequence and chromosomal structure homology with humans. Nevertheless, in comparison to other available model organisms, very little work has been devoted to pig proteomics until recently. Keeping this in mind, the present review will highlight some of the advantages and disadvantages of pig as a model system for proteomic studies.

3.1602 **Cannabinoid 1 receptor-dependent transactivation of fibroblast growth factor receptor 1 emanates from lipid rafts and amplifies extracellular signal-regulated kinase 1/2 activation in embryonic cortical neurons**
Asimaki, O., Leondaritis, G., Lois, G., Sakellaridis, N. and Mangoura, D.
J. Neurochem., ***116(5)***, *866-873 (2011)*

G-protein coupled receptors may mediate their effects on neuronal growth and differentiation through activation of extracellular signal-regulated kinases 1/2 (ERK1/2), often elicited by transactivation of growth factor receptor tyrosine kinases. This elaborate signaling process includes inducible formation and trafficking of multiprotein signaling complexes and is facilitated by pre-ordained membrane microdomains, in particular lipid rafts. In this study, we have uncovered novel signaling interactions of cannabinoid receptors with fibroblast growth factor receptors, which depended on lipid rafts and led to ERK1/2 activation in primary neurons derived from chick embryo telencephalon. More specifically, the cannabinoid 1 receptor (CB1R) agonist methanandamide induced tyrosine phosphorylation and transactivation of fibroblast growth factor receptor (FGFR)1 via Src and Fyn, which drove an amplification wave in ERK1/2 activation. Transactivation of FGFR1 was accompanied by the formation of a protein kinase C ε-dependent multiprotein complex that included CB1R, Fyn, Src, and FGFR1. Recruitment of molecules increased with time of exposure to methanandamide, suggesting that in addition to signaling it also served trafficking of receptors. Upon agonist stimulation we also detected a rapid incorporation of CB1R, as well as activated Src and Fyn, and FGFR1 in lipid rafts. Most importantly, lipid raft integrity was a pre-requisite for CB1R-dependent complex formation. Our data provide evidence that lipid rafts may organize CB1 receptor proximal signaling events, namely activation of Src and Fyn, and transactivation of FGFR1 towards activation of ERK1/2 and induction of neuronal differentiation.

3.1603 **Evidence for functional links between the Rgd1-Rho3 RhoGAP-GTPase module and Tos2, a protein involved in polarized growth in *Saccharomyces cerevisiae***
Claret, S., Roumanie, O., Prouzet-Mauleon, V., lefebre, F., Thoraval, D., Crouzet, M. and Doignon, F.
FEMS Yeast Res., ***11(2)***, *179-191 (2011)*

The Rho GTPase-activating protein Rgd1p positively regulates the GTPase activity of Rho3p and Rho4p, which are involved in bud growth and cytokinesis, respectively, in the budding yeast *Saccharomyces cerevisiae*. Two-hybrid screening identified Tos2p as a candidate Rgd1p-binding protein. Further analyses confirmed that Tos2p binds to the RhoGAP Rgd1p through its C-terminal region. Both Tos2p and Rgd1p are localized to polarized growth sites during the cell cycle and associated with detergent-resistant membranes. We observed that *TOS2* overexpression suppressed *rgd1*Δ sensitivity to a low pH. In the *tos2*Δ strain, the amount of GTP-bound Rho3p was increased, suggesting an influence of Tos2p on Rgd1p

activity *in vivo*. We also showed a functional interaction between the *TOS2* and the *RHO3* genes: *TOS2* overexpression partially suppressed the growth defect of *rho3-V51* cells at a restrictive temperature. We propose that Tos2p, a protein involved in polarized growth and most probably associated with the plasma membrane, modulates the action of Rgd1p and Rho3p in *S. cerevisiae*.

3.1604 Interaction of the Tobacco Lectin with Histone Proteins

Schouppe, D., Ghesquiere, B., Menschaert, G., De Vos, W.H., Bourque, S., Trooskens, G., Proost, P., Gevaert, K. and Van Damme, E.J.M.
*Plant Physiol., **155**, 1091-1102 (2011)*

The tobacco (*Nicotiana tabacum*) agglutinin or Nictaba is a member of a novel class of plant lectins residing in the nucleus and the cytoplasm of tobacco cells. Since tobacco lectin expression is only observed after the plant has been subjected to stress situations such as jasmonate treatment or insect attack, Nictaba is believed to act as a signaling protein involved in the stress physiology of the plant. In this paper, a nuclear proteomics approach was followed to identify the binding partners for Nictaba in the nucleus and the cytoplasm of tobacco cv Xanthi cells. Using lectin affinity chromatography and pull-down assays, it was shown that Nictaba interacts primarily with histone proteins. Binding of Nictaba with histone H2B was confirmed in vitro using affinity chromatography of purified calf thymus histone proteins on a Nictaba column. Elution of Nictaba-interacting histone proteins was achieved with 1 M *N*-acetylglucosamine (GlcNAc). Moreover, mass spectrometry analyses indicated that the Nictaba-interacting histone proteins are modified by *O*-GlcNAc. Since the lectin-histone interaction was shown to be carbohydrate dependent, it is proposed that Nictaba might fulfill a signaling role in response to stress by interacting with *O*-GlcNAcylated proteins in the plant cell nucleus.

3.1605 The Minimal Proteome in the Reduced Mitochondrion of the Parasitic Protist Giardia intestinalis

Jedelsky, P.L., Dolezal, P., Rada, P., Pyrih, J., Smid, O., Hrdy, I., Sedinova, M., Marcincikova, M., Voleman, L., Perry, A.J., Beltran, N.C., Lithgow, TS. And Tachezy, J.
*PloSOne, **6(2)**, e17285 (2011)*

The mitosomes of *Giardia intestinalis* are thought to be mitochondria highly-reduced in response to the oxygen-poor niche. We performed a quantitative proteomic assessment of *Giardia* mitosomes to increase understanding of the function and evolutionary origin of these enigmatic organelles. Mitosome-enriched fractions were obtained from cell homogenate using Optiprep gradient centrifugation. To distinguish mitosomal proteins from contamination, we used a quantitative shot-gun strategy based on isobaric tagging of peptides with iTRAQ and tandem mass spectrometry. Altogether, 638 proteins were identified in mitosome-enriched fractions. Of these, 139 proteins had iTRAQ ratio similar to that of the six known mitosomal markers. Proteins were selected for expression in *Giardia* to verify their cellular localizations and the mitosomal localization of 20 proteins was confirmed. These proteins include nine components of the FeS cluster assembly machinery, a novel diflavo-protein with NADPH reductase activity, a novel VAMP-associated protein, and a key component of the outer membrane protein translocase. None of the novel mitosomal proteins was predicted by previous genome analyses. The small proteome of the *Giardia* mitosome reflects the reduction in mitochondrial metabolism, which is limited to the FeS cluster assembly pathway, and a simplicity in the protein import pathway required for organelle biogenesis.

3.1606 The Small GTPase RhoA Localizes to the Nucleus and Is Activated by Net1 and DNA Damage Signals

Dubash,, A.D., Guilluy, C., Srougi, M.C., Boulter, E., Burridge, K. and Garcia-Mata, R.
*PloSOne, **6(2)**, e17380 (2011)*

Background
Rho GTPases control many cellular processes, including cell survival, gene expression and migration. Rho proteins reside mainly in the cytosol and are targeted to the plasma membrane (PM) upon specific activation by guanine nucleotide exchange factors (GEFs). Accordingly, most GEFs are also cytosolic or associated with the PM. However, Net1, a RhoA-specific GEF predominantly localizes to the cell nucleus at steady-state. Nuclear localization for Net1 has been seen as a mechanism for sequestering the GEF away from RhoA, effectively rendering the protein inactive. However, considering the prominence of nuclear Net1 and the fact that a biological stimulus that promotes Net1 translocation out the nucleus to the cytosol has yet to be discovered, we hypothesized that Net1 might have a previously unidentified function in the nucleus of cells.
Principal Findings

Using an affinity precipitation method to pulldown the active form of Rho GEFs from different cellular fractions, we show here that nuclear Net1 does in fact exist in an active form, contrary to previous expectations. We further demonstrate that a fraction of RhoA resides in the nucleus, and can also be found in a GTP-bound active form and that Net1 plays a role in the activation of nuclear RhoA. In addition, we show that ionizing radiation (IR) specifically promotes the activation of the nuclear pool of RhoA in a Net1-dependent manner, while the cytoplasmic activity remains unchanged. Surprisingly, irradiating isolated nuclei alone also increases nuclear RhoA activity via Net1, suggesting that all the signals required for IR-induced nuclear RhoA signaling are contained within the nucleus.
Conclusions/Significance
These results demonstrate the existence of a functional Net1/RhoA signaling pathway within the nucleus of the cell and implicate them in the DNA damage response.

3.1607 Myelination in the absence of UDP-galactose:ceramide galactosyl-transferase and fatty acid 2 – hydroxylase

Meixner, M., Jungnixkel, J., Grothe, C., Gieselmann, V. and Eckhardt, M.
*BMC Neuroscience, **12**, 22-31 (2011)*

Background
The sphingolipids galactosylceramide (GalCer) and sulfatide are major myelin components and are thought to play important roles in myelin function. The importance of GalCer and sulfatide has been validated using UDP-galactose:ceramide galactosyltransferase-deficient (*Cgt*$^{-/-}$) mice, which are impaired in myelin maintenance. These mice, however, are still able to form compact myelin. Loss of GalCer and sulfatide in these mice is accompanied by up-regulation of 2-hydroxylated fatty acid containing (HFA)-glucosylceramide in myelin. This was interpreted as a partial compensation of the loss of HFA-GalCer, which may prevent a more severe myelin phenotype. In order to test this hypothesis, we have generated *Cgt*$^{-/-}$ mice with an additional deletion of the fatty acid 2-hydroxylase (*Fa2h*) gene.
Results
Fa2h$^{-/-}$/Cgt$^{-/-}$ double-deficient mice lack sulfatide, GalCer, and in addition HFA-GlcCer and sphingomyelin. Interestingly, compared to *Cgt*$^{-/-}$ mice the amount of GlcCer in CNS myelin was strongly reduced in *Fa2h*$^{-/-}$/*Cgt*$^{-/-}$ mice by more than 80%. This was accompanied by a significant increase in sphingomyelin, which was the predominant sphingolipid in *Fa2h*$^{-/-}$/*Cgt*$^{-/-}$ mice. Despite these significant changes in myelin sphingolipids, compact myelin was formed in *Fa2h*$^{-/-}$/*Cgt*$^{-/-}$ mice, and g-ratios of myelinated axons in the spinal cord of 4-week-old *Fa2h*$^{-/-}$/*Cgt*$^{-/-}$ mice did not differ significantly from that of *Cgt*$^{-/-}$ mice, and there was no obvious phenotypic difference between *Fa2h*$^{-/-}$/*Cgt*$^{-/-}$ and *Cgt*$^{-/-}$ mice
Conclusions
These data show that compact myelin can be formed with non-hydroxylated sphingomyelin as the predominant sphingolipid and suggest that the presence of HFA-GlcCer and HFA-sphingomyelin in *Cgt*$^{-/-}$ mice does not functionally compensate the loss of HFA-GalCer.

3.1608 Norepinephrine Deficiency Is Caused by Combined Abnormal mRNA Processing and Defective Protein Trafficking of Dopamine β-Hydroxylase

Kim, C-H., Leung, A., Huh, Y.H., Yang, E., Kim, D-J., leblanc, P., Ryu, H., Kim, K., Kim, D-W., Garland, E.M., Raj, S.R., Biaggioni, I., Robertson, D. and Kim, K-S.
*J. Biol. Chem., **286(11)**, 9196-9204 (2011)*

Human norepinephrine (NE) deficiency (or dopamine β-hydroxylase (DBH) deficiency) is a rare congenital disorder of primary autonomic failure, in which neurotransmitters NE and epinephrine are undetectable. Although potential pathogenic mutations, such as a common splice donor site mutation (IVS1+2T→C) and various missense mutations, in NE deficiency patients were identified, molecular mechanisms underlying this disease remain unknown. Here, we show that the IVS1+2T→C mutation results in a non-detectable level of DBH protein production and that all three missense mutations tested lead to the DBH protein being trapped in the endoplasmic reticulum (ER). Supporting the view that mutant DBH induces an ER stress response, exogenous expression of mutant DBH dramatically induced expression of BiP, a master ER chaperone. Furthermore, we found that a pharmacological chaperone, glycerol, significantly rescued defective trafficking of mutant DBH proteins. Taken together, we propose that NE deficiency is caused by the combined abnormal processing of DBH mRNA and defective protein trafficking and that this disease could be treated by a pharmacological chaperone(s).

3.1609 Affinity of PIP-aquaporins to sterol-enriched domains in plasma membrane of the cells of etiolated pea seedlings

Belugin, B.V., Zhestkova, I.M. and Trofimova, M.S.

*Membrane and Cell Biology, **5(1)**, 56-63 (2011)*

The hypothesis that sterol-enriched domains represent sites of preferred localization of PIP-aquaporins was tested in experiments on plasma membranes isolated from cells of etiolated pea (*Pisum sativum* L.) seedlings. Plasma membranes were isolated from microsomes by the partition in the aqueous two-phase polymer system and separated into vesicle fractions of different buoyant density by flotation in discontinuous OptiPrep gradient. Two types of plasma membrane preparations were used: one was treated with cold 1% Triton X-100 and the other was not. In untreated preparations, three populations of plasma membrane vesicles were obtained, while in the case of treated preparations, fractions of detergent-resistant membranes (DRM) and solubilized membrane proteins were obtained. In all membrane fractions collected after OptiPrep flotation, the amounts of proteins, sterols, and PIP-aquaporins were determined. The highest sterol content was detected in the membrane fraction with buoyant density 1.098 g/cm^3 and in the DRM fraction (1.146 g/cm^3). These fractions contained much more PIP-aquaporins than the other ones. Phase state of the lipid bilayer was determined by measuring generalized polarization excitation of fluorescence (GPEX) of laurdan incorporated into the membranes of different fractions. It was revealed that the lipid bilayer of the membranes with density of 1.098 g/cm^3 had a higher extent of ordering than that of the fractions with density of ~ 1.146 g/cm^3. The results indicated that uppermost local concentrations of PIP-aquaporins were associated with tightly packed sterol-enriched domains. Moreover, upon solubilization of plasma membrane with Triton X-100, PIP-aquaporins mainly resided in DRM, thus exhibiting a high affinity to sterols.

3.1610 Proteomic Analysis of the Pancreatic Islet β-Cell Secretory Granule: Current Understanding and Future Opportunities

Cooper, G.J.S.

*Systems Biol., **2(3)**, 327-362 (2011)*

The pancreatic islet β-cell granule has been the subject of intense study for decades, in part because it serves as the vehicle for the regulated secretion of insulin and amylin , through which it exerts regulation of metabolism. β-cell granule proteins have been closely linked to disease mechanisms in both major types of diabetes, and recent findings from genome-wide association studies have reinforced the importance of these linkages for understanding disease mechanisms . Granule proteins have also proven to be of major interest in pharmaceutics, since two of them, insulin and amylin , have each served as the basis for the development of anti-diabetic pharmacotherapies . In spite of all the attention this enigmatic granule has received to date, many fundamental questions about its molecular structure and function remain unanswered. In the past few years, high-resolution methodologies have begun to unravel the granule proteome in ever-increasing detail. Emerging data complement the results from the other approaches that have been applied to understand the granule. This chapter will explore the current state of knowledge in the field and the implications of emerging proteomic data for the study of physiological processes and disease mechanisms in diabetes.

3.1611 Adenosine receptors and membrane microdomains

Laskey, R.D.

*Biochim. Biophys. Acta, **1808**, 1284-1289 (2011)*

Adenosine receptors are a member of the large family of seven transmembrane spanning G protein coupled receptors. The four adenosine receptor subtypes—A1, A2a, A2b, A3—exert their effects via the activation of one or more heterotrimeric G proteins resulting in the modulation of intracellular signaling. Numerous studies over the past decade have documented the complexity of G protein coupled receptor signaling at the level of protein–protein interactions as well as through signaling cross talk. With respect to adenosine receptors, the activation of one receptor subtype can have profound direct effects in one cell type but little or no effect in other cells. There is significant evidence that the compartmentation of subcellular signaling plays a physiological role in the fidelity of G protein coupled receptor signaling. This compartmentation is evident at the level of the plasma membrane in the form of membrane microdomains such as caveolae and lipid rafts. This review will summarize and critically assess our current understanding of the role of membrane microdomains in regulating adenosine receptor signaling.

3.1612 A Point Mutation in the Amino Terminus of TLR7 Abolishes Signaling without Affecting Ligand Binding

Iavarone, C., Ramsauer, K., Kubarenko, A.V., Debasitis, J.C., Leukin, I., Weber, A.N.R., Siggs, O.M., Beutler, B., Zhang, P., Otten, g., D'Oro, U., Valiante, N.M., Mbow, M.L. and Vissintin, A.
*J. Immunol., **186**, 4213-4222 (2011)*

TLR7 is the mammalian receptor for ssRNA and some nucleotide-like small molecules. We have generated a mouse by N-nitrose-N′-ethyl urea mutagenesis in which threonine 68 of TLR7 was substituted with isoleucine. Cells bearing this mutant TLR7 lost the sensitivity to the small-molecule TLR7 agonist resiquimod, hence the name TLR7^{rsq1}. In this work, we report the characterization of this mutant protein. Similar to the wild-type counterpart, TLR7^{rsq1} localizes to the endoplasmic reticulum and is expressed at normal levels in both primary cells and reconstituted 293T cells. In addition to small-molecule TLR7 agonists, TLR7^{rsq1} fails to be activated by ssRNA. Whole-transcriptome analysis demonstrates that TLR7 is the exclusive and indispensable receptor for both classes of ligands, consistent with the fact that both ligands induce highly similar transcriptional signatures in TLR7$^{wt/wt}$ splenocytes. Thus, TLR7^{rsq1} is a bona fide phenocopy of the TLR7 null mouse. Because TLR7^{rsq1} binds to ssRNA, our studies imply that the N-terminal portion of TLR7 triggers a yet to be identified event on TLR7. TLR7^{rsq1} mice might represent a valuable tool to help elucidate novel aspects of TLR7 biology.

3.1613 Reciprocal Control of hERG Stability by Hsp70 and Hsc70 With Implication for Restoration of LQT2 Mutant Stability

Li, P.L., Ninomiya, H., Kurata, Y., Kato, M., Miake, J., Yamamoto, Y., Igawa, O., Nakai, A., Higaki, K., Toyoda, F., Wu, J., Horie, M., Matsuura, H., Yoshida, A., Shirayoshi, Y., Hiraoka, M. and Hisatome, I.
*Circ. Res., **108**, 458-468 (2011)*

Rationale:The human ether-a-go-go–related gene (hERG) encodes the α subunit of the potassium current I_{Kr}. It is highly expressed in cardiomyocytes and its mutations cause long QT syndrome type 2. Heat shock protein (Hsp)70 is known to promote maturation of hERG. Hsp70 and heat shock cognate (Hsc70) 70 has been suggested to play a similar function. However, Hsc70 has recently been reported to counteract Hsp70.
Objective:We investigated whether Hsc70 counteracts Hsp70 in the control of wild-type and mutant hERG stability.
Methods and Results:Coexpression of Hsp70 with hERG in HEK293 cells suppressed hERG ubiquitination and increased the levels of both immature and mature forms of hERG. Immunocytochemistry revealed increased levels of hERG in the endoplasmic reticulum and on the cell surface. Electrophysiological studies showed increased I_{Kr}. All these effects of Hsp70 were abolished by Hsc70 coexpression. Heat shock treatment of HL-1 mouse cardiomyocytes induced endogenous Hsp70, switched mouse ERG associated with Hsc70 to Hsp70, increased I_{Kr}, and shortened action potential duration. Channels with disease-causing missense mutations in intracellular domains had a higher binding capacity to Hsc70 than wild-type channels and channels with mutations in the pore region. Knockdown of Hsc70 by small interfering RNA or heat shock prevented degradation of mutant hERG proteins with mutations in intracellular domains.
Conclusions:These results indicate reciprocal control of hERG stability by Hsp70 and Hsc70. Hsc70 is a potential target in the treatment of LQT2 resulting from missense hERG mutations.

3.1614 Specific elimination of effector memory CD4+ T cells due to enhanced Fas signaling complex formation and association with lipid raft microdomains

Ramaswamy, M., Cruz, A.C., Cleland, S.Y., Deng, M., Price, S., Rao, V.K. and Siegel, R.M.
*Cell Death and Differentiation, **18**, 712-720 (2011)*

Elimination of autoreactive CD4⁺ T cells through the death receptor Fas/CD95 is an important mechanism of immunological self-tolerance. Fas deficiency results in systemic autoimmunity, yet does not affect the kinetics of T-cell responses to acute antigen exposure or infection. Here we show that Fas and TCR-induced apoptosis are largely restricted to CD4⁺ T cells with an effector memory phenotype (effector memory T cells (T_{EM})), whereas central memory and activated naïve CD4⁺ T cells are relatively resistant to both. Sensitivity of T_{EM} to Fas-induced apoptosis depends on enrichment of Fas in lipid raft microdomains, and is linked to more efficient formation of the Fas death-inducing signaling complex. These results explain how Fas can cull T cells reactive against self-antigens without affecting acute

immune responses. This work also identifies Fas-induced apoptosis as a possible immunotherapeutic strategy to eliminate T_{EM} linked to the pathogenesis of a number of autoimmune diseases.

3.1615 Docosahexaenoic Acid Reduces Amyloid β Production via Multiple Pleiotropic Mechanisms
Grimm, M.O., Kuchenbecker, J., Grôsgen, S., Burg, V.K., Hundsdôrfer, B., Rothhaar, T.L., Friess, P., de Wilde, M.C., Broersen, L.M., Penke, B., Peter, M., Vigh, L., Grimm, H.S. and Hartmann, T.
J. Biol. Chem., ***286(16)***, *14028-14039 (2011)*

Alzheimer disease is characterized by accumulation of the β-amyloid peptide (Aβ) generated by β- and γ-secretase processing of the amyloid precursor protein (APP). The intake of the polyunsaturated fatty acid docosahexaenoic acid (DHA) has been associated with decreased amyloid deposition and a reduced risk in Alzheimer disease in several epidemiological trials; however, the exact underlying molecular mechanism remains to be elucidated. Here, we systematically investigate the effect of DHA on amyloidogenic and nonamyloidogenic APP processing and the potential cross-links to cholesterol metabolism *in vivo* and *in vitro*. DHA reduces amyloidogenic processing by decreasing β- and γ-secretase activity, whereas the expression and protein levels of BACE1 and presenilin1 remain unchanged. In addition, DHA increases protein stability of α-secretase resulting in increased nonamyloidogenic processing. Besides the known effect of DHA to decrease cholesterol *de novo* synthesis, we found cholesterol distribution in plasma membrane to be altered. In the presence of DHA, cholesterol shifts from raft to non-raft domains, and this is accompanied by a shift in γ-secretase activity and presenilin1 protein levels. Taken together, DHA directs amyloidogenic processing of APP toward nonamyloidogenic processing, effectively reducing Aβ release. DHA has a typical pleiotropic effect; DHA-mediated Aβ reduction is not the consequence of a single major mechanism but is the result of combined multiple effects.

3.1616 Disease-associated GPR56 Mutations Cause Bilateral Frontoparietal Polymicrogyria via Multiple Mechanisms
Chiang, N-Y., Hsiao, C-C., Huang, Y-S., Chen, H-Y., Hsieh, I-J., Chang, G-W. and Lin, H-H.
J. Biol. Chem., ***286(16)***, *14215-14225 (2011)*

Loss-of-function mutations in the gene encoding G protein-coupled receptor 56 (GPR56) lead to bilateral frontoparietal polymicrogyria (BFPP), an autosomal recessive disorder affecting brain development. The GPR56 receptor is a member of the adhesion-GPCR family characterized by the chimeric composition of a long ectodomain (ECD), a GPCR proteolysis site (GPS), and a seven-pass transmembrane (7TM) moiety. Interestingly, all identified BFPP-associated missense mutations are located within the extracellular region of GPR56 including the ECD, GPS, and the extracellular loops of 7TM. In the present study, a detailed molecular and functional analysis of the wild-type GPR56 and BFPP-associated point mutants shows that individual GPR56 mutants most likely cause BFPP via different combination of multiple mechanisms. These include reduced surface receptor expression, loss of GPS proteolysis, reduced receptor shedding, inability to interact with a novel protein ligand, and differential distribution of the 7TM moiety in lipid rafts. These results provide novel insights into the cellular functions of GPR56 receptor and reveal molecular mechanisms whereby GPR56 mutations induce BFPP.

3.1617 CIN85 Modulates the Down-regulation of Fcγ RIIa Expression and Function by c-Cbl in a PKC-dependent Manner in Human Neutrophils
Marois, L., Vaillancourt, M., Pare, G., Gagne, V., Fernandes, M.J.G., Rollet-Labelle, E. and Naccache, P.H.
J. Biol. Chem., ***286(17)***, *15073-15084 (2011)*

We previously described a non-classical mechanism that arrests FcγRIIa signaling in human neutrophils once engaged by immune complexes or opsonized pathogens. The engagement of FcγRIIa leads to its ubiquitination by the ubiquitin ligase c-Cbl and degradation by the proteasome. Herein, we further examined some of the events regulating this novel pathway. The adaptor protein CIN85 was described in other systems to be involved in the regulation of the c-Cbl-dependent pathway. We found that CIN85 is expressed in human neutrophils and that it translocates like c-Cbl from the cytosol to the plasma membrane following receptor cross-linking. CIN85 was also recruited to the same subset of high density detergent-resistant membrane fractions in which stimulated FcγRIIa partitioned with c-Cbl. The integrity of these microdomains is essential to the FcγRIIa degradation process because the cholesterol-depleting agent methyl-β-cyclodextrin inhibits this event. Silencing the expression of CIN85 by siRNA in dibutyryl cyclic AMP-differentiated PLB 985 cells prevented FcγRIIa degradation and increased IgG-mediated phagocytosis. Confocal microscopy revealed that the presence of CIN85 is essential to the proper sorting

of FcγRIIa during endocytosis. We also provide direct evidence that CIN85 is a substrate of serine/threonine kinase PKCs. Classical PKCs positively regulate FcγRIIa ubiquitination and degradation because these events were inhibited by Gö6976, a classical PKC inhibitor. We conclude that the ubiquitination and degradation of stimulated FcγRIIa mediated by c-Cbl are positively regulated by the adaptor protein CIN85 in a PKC-dependent manner and that these events contribute to the termination of FcγRIIa signaling.

3.1618 Advances in qualitative and quantitative plant membrane proteomics
Kota, U. and Goshe, M.B.
Phytochemistry, ***72***, *1040-1060 (2011)*

The membrane proteome consists of integral and membrane-associated proteins that are involved in various physiological and biochemical functions critical for cellular function. It is also dynamic in nature, where many proteins are only expressed during certain developmental stages or in response to environmental stress. These proteins can undergo post-translational modifications in response to these different conditions, allowing them to transiently associate with the membrane or other membrane proteins. Along with their increased size, hydrophobicity, and the additional organelle and cellular features of plant cells relative to mammalian systems, the characterization of the plant membrane proteome presents unique challenges for effective qualitative and quantitative analysis using mass spectrometry (MS) analysis. Here, we present the latest advancements developed for the isolation and fractionation of plant organelles and their membrane components amenable to MS analysis. Separations of membrane proteins from these enriched preparations that have proven effective are discussed for both gel- and liquid chromatography-based MS analysis. In this context, quantitative membrane proteomic analyses using both isotope-coded and label-free approaches are presented and reveal the potential to establish a wider-biological interpretation of the function of plant membrane proteins that will ultimately lead to a more comprehensive understanding of plant physiology and their response mechanisms.

3.1619 FERM Domain Phosphoinositide Binding Targets Merlin to the Membrane and Is Essential for Its Growth-Suppressive Function
Mani, T., Hennigan, R.F., Foster, L.A., Conrady, D.G., Herr, A.B. and Ip, W.
Mol. Cell. Biol., ***31(10)***, *1983-1996 (2011)*

The neurofibromatosis type 2 tumor suppressor protein, merlin, is related to the ERM (ezrin, radixin, and moesin) family of plasma membrane-actin cytoskeleton linkers. For ezrin, phosphatidylinositol 4,5-bisphosphate (PIP2) binding to the amino-terminal FERM domain is required for its conformational activation, proper subcellular localization, and function, but less is known about the role of phosphoinositide binding for merlin. Current evidence indicates that association with the membrane is important for merlin to function as a growth regulator; however, the mechanisms by which merlin localizes to the membrane are less clear. Here, we report that merlin binds phosphoinositides, including PIP2, via a conserved binding motif in its FERM domain. Abolition of FERM domain-mediated phosphoinositide binding of merlin displaces merlin from the membrane and releases it into the cytosol without altering the folding of merlin. Importantly, a merlin protein whose FERM domain cannot bind phosphoinositide is defective in growth suppression. Retargeting the mutant merlin into the membrane using a dual-acylated amino-terminal decapeptide from Fyn is sufficient to restore the growth-suppressive properties to the mutant merlin. Thus, FERM domain-mediated phosphoinositide binding and membrane association are critical for the growth-regulatory function of merlin.

3.1620 Motor Protein Myo1c Is a Podocyte Protein That Facilitates the Transport of Slit Diaphragm Protein Neph1 to the Podocyte Membrane
Arif, E., Wagner, M.C., Johnstone, D.B., Wong, H.N., George, W.B., Pruthi, P.A., Lazzara, M.J. and Nihalani, D.
Mol. Cell. Biol., ***31(10)***, *2134-2150 (2011)*

The podocyte proteins Neph1 and nephrin organize a signaling complex at the podocyte cell membrane that forms the structural framework for a functional glomerular filtration barrier. Mechanisms regulating the movement of these proteins to and from the membrane are currently unknown. This study identifies a novel interaction between Neph1 and the motor protein Myo1c, where Myo1c plays an active role in targeting Neph1 to the podocyte cell membrane. Using in vivo and in vitro experiments, we provide data

supporting a direct interaction between Neph1 and Myo1c which is dynamic and actin dependent. Unlike wild-type Myo1c, the membrane localization of Neph1 was significantly reduced in podocytes expressing dominant negative Myo1c. In addition, Neph1 failed to localize at the podocyte cell membrane and cell junctions in Myo1c-depleted podocytes. We further demonstrate that similarly to Neph1, Myo1c also binds nephrin and reduces its localization at the podocyte cell membrane. A functional analysis of Myo1c knockdown cells showed defects in cell migration, as determined by a wound assay. In addition, the ability to form tight junctions was impaired in Myo1c knockdown cells, as determined by transepithelial electric resistance (TER) and bovine serum albumin (BSA) permeability assays. These results identify a novel Myo1c-dependent molecular mechanism that mediates the dynamic organization of Neph1 and nephrin at the slit diaphragm and is critical for podocyte function.

3.1621 ***Drosophila*** **Photoreceptor Cells Exploited for the Production of Eukaryotic Membrane Proteins: Receptors, Transporters and Channels**
Panneels, V., Kock, I., Krijnse-Locker, J., Rezgaoui, M. and Sinning, I.
PloSOne, ***6(4)****, e18478 (2011)*

Background
Membrane proteins (MPs) play key roles in signal transduction. However, understanding their function at a molecular level is mostly hampered by the lack of protein in suitable amount and quality. Despite impressive developments in the expression of prokaryotic MPs, eukaryotic MP production has lagged behind and there is a need for new expression strategies. In a pilot study, we produced a *Drosophila* glutamate receptor specifically in the eyes of transgenic flies, exploiting the naturally abundant membrane stacks in the photoreceptor cells (PRCs). Now we address the question whether the PRCs also process different classes of medically relevant target MPs which were so far notoriously difficult to handle with conventional expression strategies.
Principal Findings
We describe the homologous and heterologous expression of 10 different targets from the three major MP classes - G protein-coupled receptors (GPCRs), transporters and channels in *Drosophila* eyes. PRCs offered an extraordinary capacity to produce, fold and accommodate massive amounts of MPs. The expression of some MPs reached similar levels as the endogenous rhodopsin, indicating that the PRC membranes were almost unsaturable. Expression of endogenous rhodopsin was not affected by the target MPs and both could coexist in the membrane stacks. Heterologous expression levels reached about 270 to 500 pmol/mg total MP, resulting in 0.2–0.4 mg purified target MP from 1 g of fly heads. The metabotropic glutamate receptor and human serotonin transporter - both involved in synaptic transmission - showed native pharmacological characteristics and could be purified to homogeneity as a prerequisite for further studies.
Significance
We demonstrate expression in *Drosophila* PRCs as an efficient and inexpensive tool for the large scale production of functional eukaryotic MPs. The fly eye system offers a number of advantages over conventional expression systems and paves the way for in-depth analyses of eukaryotic MPs that have so far not been accessible to biochemical and biophysical studies.

3.1622 **Calcium Uptake and Proton Transport by Acidocalcisomes of** ***Toxoplasma gondii***
Rohloff, P., Miranda, K., Rodrigues, J.F.C., Fang, J., Galizzi, M., Plattner, H., Hentschel, J. and Moreno, S.N.J.
PloSOne, ***6(4)****, e18390 (2011)*

Acidocalcisomes are acidic calcium stores found in diverse organisms, being conserved from bacteria to humans. They possess an acidic matrix that contains several cations bound to phosphates, which are mainly present in the form of short and long polyphosphate chains. Their matrix is acidified through the action of proton pumps such as a vacuolar proton ATPase and a vacuolar proton pyrophosphatase. Calcium uptake occurs through a Ca^{2+}/H^{+} countertransporting ATPase located in the membrane of the organelle. Acidocalcisomes have been identified in a variety of microorganisms, including Apicomplexan parasites such as *Plasmodium* and *Eimeria* species, and in *Toxoplasma gondii*. We report the purification and characterization of an acidocalcisome fraction from *T. gondii* tachyzoites after subcellular fractionation and further discontinuous iodixanol gradient purification. Proton and calcium transport activities in the fraction were characterized by fluorescence microscopy and spectrophotometric methods using acridine orange and arsenazo III, respectively. This work will facilitate the understanding of the function of acidocalcisomes in Apicomplexan parasites, as we can now isolate highly purified fractions that could be used for proteomic analysis to find proteins that may clarify the biogenesis of these organelles.

3.1623 **The Expression of a Xylanase Targeted to ER-Protein Bodies Provides a Simple Strategy to Produce Active Insoluble Enzyme Polymers in Tobacco Plants**
Llop-Tous, I., Ortiz, M., Torrent, M. and Ludevid, M.D.
PloSOne, ***6(4)***, *e19474 (2011)*

Background
Xylanases deserve particular attention due to their potential application in the feed, pulp bleaching and paper industries. We have developed here an efficient system for the production of an active xylanase in tobacco plants fused to a proline-rich domain (Zera) of the maize storage protein γ-zein. Zera is a self-assembling domain able to form protein aggregates *in vivo* packed in newly formed endoplasmic reticulum-derived organelles known as protein bodies (PBs).
Methodology/Principal Findings
Tobacco leaves were transiently transformed with a binary vector containing the Zera-xylanase coding region, which was optimized for plant expression, under the control of the 35S CaMV promoter. The fusion protein was efficiently expressed and stored in dense PBs, resulting in yields of up to 9% of total protein. Zera-xylanase was post-translationally modified with high-mannose-type glycans. Xylanase fused to Zera was biologically active not only when solubilized from PBs but also in its insoluble form. The resistance of insoluble Zera-xylanase to trypsin digestion demonstrated that the correct folding of xylanase in PBs was not impaired by Zera oligomerization. The activity of insoluble Zera-xylanase was enhanced when substrate accessibility was facilitated by physical treatments such as ultrasound. Moreover, we found that the thermostability of the enzyme was improved when Zera was fused to the C-terminus of xylanase.
Conclusion/Significance
In the present work we have successfully produced an active insoluble aggregate of xylanase fused to Zera in plants. Zera-xylanase chimeric protein accumulates within ER-derived protein bodies as active aggregates that can easily be recovered by a simple density-based downstream process. The production of insoluble active Zera-xylanase protein in tobacco outlines the potential of Zera as a fusion partner for producing enzymes of biotechnological relevance. Zera-PBs could thus become efficient and low-cost bioreactors for industrial purposes.

3.1624 **Identification of Contractile Vacuole Proteins in Trypanosoma cruzi**
Ulrich, P.N., Jimenez, V., Park, M., Martins, V.P., Atwood III, J., Moles, K., Collins, D., Rohloff, P., Tarleton, R., Moreno, S.N.J., Orlando, R. and Docampo, R.
PloSOne, ***6(3)***, *e18013 (2011)*

Contractile vacuole complexes are critical components of cell volume regulation and have been shown to have other functional roles in several free-living protists. However, very little is known about the functions of the contractile vacuole complex of the parasite *Trypanosoma cruzi*, the etiologic agent of Chagas disease, other than a role in osmoregulation. Identification of the protein composition of these organelles is important for understanding their physiological roles. We applied a combined proteomic and bioinfomatic approach to identify proteins localized to the contractile vacuole. Proteomic analysis of a *T. cruzi* fraction enriched for contractile vacuoles and analyzed by one-dimensional gel electrophoresis and LC-MS/MS resulted in the addition of 109 newly detected proteins to the group of expressed proteins of epimastigotes. We also identified different peptides that map to at least 39 members of the dispersed gene family 1 (DGF-1) providing evidence that many members of this family are simultaneously expressed in epimastigotes. Of the proteins present in the fraction we selected several homologues with known localizations in contractile vacuoles of other organisms and others that we expected to be present in these vacuoles on the basis of their potential roles. We determined the localization of each by expression as GFP-fusion proteins or with specific antibodies. Six of these putative proteins (Rab11, Rab32, AP180, ATPase subunit B, VAMP1, and phosphate transporter) predominantly localized to the vacuole bladder. TcSNARE2.1, TcSNARE2.2, and calmodulin localized to the spongiome. Calmodulin was also cytosolic. Our results demonstrate the utility of combining subcellular fractionation, proteomic analysis, and bioinformatic approaches for localization of organellar proteins that are difficult to detect with whole cell methodologies. The CV localization of the proteins investigated revealed potential novel roles of these organelles in phosphate metabolism and provided information on the potential participation of adaptor protein complexes in their biogenesis.

3.1625 **A *Pseudomonas aeruginosa* Toxin that Hijacks the Host Ubiquitin Proteolytic System**
Bomberger, J.M., Ye, S., MacEachran, D.P., Koeppen, K., Barnaby, R.L., O'Toole, G. and Stanton, B.A.
PloSPathogens, ***7(3)***, *e1001325 (2011)*

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic pathogen chronically infecting the lungs of patients with chronic obstructive pulmonary disease (COPD), pneumonia, cystic fibrosis (CF), and bronchiectasis. Cif (PA2934), a bacterial toxin secreted in outer membrane vesicles (OMV) by *P. aeruginosa*, reduces CFTR-mediated chloride secretion by human airway epithelial cells, a key driving force for mucociliary clearance. The aim of this study was to investigate the mechanism whereby Cif reduces CFTR-mediated chloride secretion. Cif redirected endocytosed CFTR from recycling endosomes to lysosomes by stabilizing an inhibitory effect of G3BP1 on the deubiquitinating enzyme (DUB), USP10, thereby reducing USP10-mediated deubiquitination of CFTR and increasing the degradation of CFTR in lysosomes. This is the first example of a bacterial toxin that regulates the activity of a host DUB. These data suggest that the ability of *P. aeruginosa* to chronically infect the lungs of patients with COPD, pneumonia, CF, and bronchiectasis is due in part to the secretion of OMV containing Cif, which inhibits CFTR-mediated chloride secretion and thereby reduces the mucociliary clearance of pathogens.

3.1626 Tandem application of cationic colloidal silica and Triton X-114 for plasma membrane protein isolation and purification: Towards developing an MDCK protein database

Mathias, R.A., Chen, Y-S., Goode, R.J.A., Kapp, E.A., Mathivanan, S., Moritz, R.L., Zhu, H-J. and Simpson, R.J.

*Proteomics, **11**, 1238-1253 (2011)*

Plasma membrane (PM) proteins are attractive therapeutic targets because of their accessibility to drugs. Although genes encoding PM proteins represent 20–30% of eukaryotic genomes, a detailed characterisation of their encoded proteins is underrepresented, due, to their low copy number and the inherent difficulties in their isolation and purification as a consequence of their high hydrophobicity. We describe here a strategy that combines two orthogonal methods to isolate and purify PM proteins from Madin Darby canine kidney (MDCK) cells. In this two-step method, we first used cationic colloidal silica (CCS) to isolate adherent (Ad) and non-adherent (nAd) PM fractions, and then subjected each fraction to Triton X-114 (TX-114) phase partitioning to further enrich for hydrophobic proteins. While CCS alone identified 255/757 (34%) membrane proteins, CCS/TX-114 in combination yielded 453/745 (61%). Strikingly, of those proteins unique to CCS/TX-114, 277/393 (70%) had membrane annotation. Further characterisation of the CCS/TX-114 data set using Uniprot and transmembrane hidden Markov model revealed that 306/745 (41%) contained one or more transmembrane domains (TMDs), including proteins with 25 and 17 TMDs. Of the remaining proteins in the data set, 69/439 (16%) are known to contain lipid modifications. Of all membrane proteins identified, 93 had PM origin, including proteins that mediate cell adhesion, modulate transmembrane ion transport, and cell–cell communication. These studies reveal that the application of CCS to first isolate Ad and nAd PM fractions, followed by their detergent-phase TX-114 partitioning, to be a powerful method to isolate low-abundance PM proteins, and a useful adjunct for in-depth cell surface proteome analyses.

3.1627 Toward a definition of the complete proteome of plant peroxisomes: Where experimental proteomics must be complemented by bioinformatics

Reumann, S.

*Proteomics, **11**, 1764-1779 (2011)*

In the past few years, proteome analysis of *Arabidopsis* peroxisomes has been established by the complementary efforts of four research groups and has emerged as the major unbiased approach to identify new peroxisomal proteins on a large scale. Collectively, more than 100 new candidate proteins from plant peroxisomes have been identified, including long-awaited low-abundance proteins. More than 50 proteins have been validated as peroxisome targeted, nearly doubling the number of established plant peroxisomal proteins. Sequence homologies of the new proteins predict unexpected enzyme activities, novel metabolic pathways and unknown non-metabolic peroxisome functions. Despite this remarkable success, proteome analyses of plant peroxisomes remain highly material intensive and require major preparative efforts. Characterization of the membrane proteome or post-translational protein modifications poses major technical challenges. New strategies, including quantitative mass spectrometry methods, need to be applied to allow further identifications of plant peroxisomal proteins, such as of stress-inducible proteins. In the long process of defining the complete proteome of plant peroxisomes, the prediction of peroxisome-targeted proteins from plant genome sequences emerges as an essential complementary approach to identify additional peroxisomal proteins that are, for instance, specific to peroxisome variants from minor tissues and organs or to abiotically stressed model and crop plants.

3.1628 Transient receptor potential canonical channels are essential for chemotactic migration of human malignant gliomas

Bomben, V.C., Turner, K.L., Barclay, T-T.C. and Sontheimer, H.
J. Cell. Physiol., ***226***, *1879-1888 (2011)*

The majority of malignant primary brain tumors are gliomas, derived from glial cells. Grade IV gliomas, Glioblastoma multiforme, are extremely invasive and the clinical prognosis for patients is dismal. Gliomas utilize a number of proteins and pathways to infiltrate the brain parenchyma including ion channels and calcium signaling pathways. In this study, we investigated the localization and functional relevance of transient receptor potential canonical (TRPC) channels in glioma migration. We show that gliomas are attracted in a chemotactic manner to epidermal growth factor (EGF). Stimulation with EGF results in TRPC1 channel localization to the leading edge of migrating D54MG glioma cells. Additionally, TRPC1 channels co-localize with the lipid raft proteins, caveolin-1 and β-cholera toxin, and biochemical assays show TRPC1 in the caveolar raft fraction of the membrane. Chemotaxis toward EGF was lost when TRPC channels were pharmacologically inhibited or by shRNA knockdown of TRPC1 channels, yet without affecting unstimulated cell motility. Moreover, lipid raft integrity was required for gliomas chemotaxis. Disruption of lipid rafts not only impaired chemotaxis but also impaired TRPC currents in whole cell recordings and decreased store-operated calcium entry as revealed by ratiomeric calcium imaging. These data indicated that TRPC1 channel association with lipid rafts is essential for glioma chemotaxis in response to stimuli, such as EGF.

3.1629 Sphingosine 1-Phosphate-Induced Motility and Endocytosis of Dendritic Cells Is Regulated by SWAP-70 through RhoA

Ocana-Morgner, C., Reichardt, P., Chopin, M., Braungart, S., Wahren, C., Gunzer, M. and Jessberger, R.
J. Immunol., ***186***, *5345-5355 (2011)*

The phospholipid mediator sphingosine 1-phosphate (S1P) enhances motility and endocytosis of mature dendritic cells (DCs). We show that in vitro migration of *Swap-70*$^{-/-}$ bone marrow-derived DCs (BMDCs) in response to S1P and S1P-induced upregulation of endocytosis are significantly reduced. S1P-stimulated movement of *Swap-70*$^{-/-}$ BMDCs, specifically retraction of their trailing edge, in a collagen three-dimensional environment is impaired. These in vitro observations correlate with delayed entry into lymphatic vessels and migration to lymph nodes of skin DCs in *Swap-70*$^{-/-}$ mice. Expression of S1P receptors ($S1P_{1–3}$) by wild-type and *Swap-70*$^{-/-}$ BMDCs is similar, but *Swap-70*$^{-/-}$ BMDCs fail to activate RhoA and to localize Rac1 and RhoA into areas of actin polymerization after S1P stimulus. The Rho-activating G protein $G\alpha_i$ interacts with SWAP-70, which also supports the localization of $G\alpha_{13}$ to membrane rafts in BMDCs. LPS-matured *Swap-70*$^{-/-}$ BMDCs contain significantly more active RhoA than wild-type DCs. Preinhibition of Rho activation restored migration to S1P, S1P-induced upregulation of endocytosis in mature *Swap-70*$^{-/-}$ BMDCs, and localization of $G\alpha_{13}$ to membrane rafts. These data demonstrate SWAP-70 as a novel regulator of S1P signaling necessary for DC motility and endocytosis.

3.1630 Cutting Edge: NKG2D-Dependent Cytotoxicity Is Controlled by Ligand Distribution in the Target Cell Membrane

Martinez, E., Brzostowski, J.A., Long, E.O. and Gross, C.C.
J. Immunol., ***186***, *5538-5542 (2011)*

Although the importance of membrane microdomains in receptor-mediated activation of lymphocytes has been established, much less is known about the role of receptor ligand distribution on APC and target cells. Detergent-resistant membrane domains, into which GPI-linked proteins partition, are enriched in cholesterol and glycosphingolipids. ULBP1 is a GPI-linked ligand for natural cytotoxicity receptor NKG2D. To investigate how ULBP1 distribution on target cells affects NKG2D-dependent NK cell activation, we fused the extracellular domain of ULBP1 to the transmembrane domain of CD45. Introduction of this transmembrane domain eliminated the association of ULBP1 with the detergent-resistant membrane fraction and caused a significant reduction of cytotoxicity and degranulation by NK cells. Clustering and lateral diffusion of ULBP1 was not affected by changes in the membrane anchor. These results show that the partitioning of receptor ligands in discrete membrane domains of target cells is an important determinant of NK cell activation.

3.1631 Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells
Weichhart, T., Haidinger, M., Katholnig, K., Kopecky, C., poglitsch, M., Lassnig, C., Rosner, M., Zlabinger, G.J., Hengstschlâger, M., Müller, M., Hörl, W.H. and Säemann, M.D.
Blood, ***117(16)****, 4273-4283 (2011)*

A central role for the mammalian target of rapamycin (mTOR) in innate immunity has been recently defined by its ability to limit proinflammatory mediators. Although glucocorticoids (GCs) exert potent anti-inflammatory effects in innate immune cells, it is currently unknown whether the mTOR pathway interferes with GC signaling. Here we show that inhibition of mTOR with rapamycin or Torin1 prevented the anti-inflammatory potency of GC both in human monocytes and myeloid dendritic cells. GCs could not suppress nuclear factor-κB and JNK activation, the expression of proinflammatory cytokines, and the promotion of Th1 responses when mTOR was inhibited. Interestingly, long-term activation of monocytes with lipopolysaccharide enhanced the expression of TSC2, the principle negative regulator of mTOR, whereas dexamethasone blocked TSC2 expression and reestablished mTOR activation. Renal transplant patients receiving rapamycin but not those receiving calcineurin inhibitors displayed a state of innate immune cell hyper-responsiveness despite the concurrent use of GC. Finally, mTOR inhibition was able to override the healing phenotype of dexamethasone in a murine lipopolysaccharide shock model. Collectively, these data identify a novel link between the glucocorticoid receptor and mTOR in innate immune cells, which is of considerable clinical importance in a variety of disorders, including allogeneic transplantation, autoimmune diseases, and cancer.

3.1632 Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes
Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S. and Wood, M.J.A.
Nature Biotech., ***29(4)****, 341-345 (2011)*

To realize the therapeutic potential of RNA drugs, efficient, tissue-specific and nonimmunogenic delivery technologies must be developed. Here we show that exosomes—endogenous nano-vesicles that transport RNAs and proteins[1, 2]—can deliver short interfering (si)RNA to the brain in mice. To reduce immunogenicity, we used self-derived dendritic cells for exosome production. Targeting was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide[3]. Purified exosomes were loaded with exogenous siRNA by electroporation. Intravenously injected RVG-targeted exosomes delivered *GAPDH* siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of *BACE1*, a therapeutic target in Alzheimer's disease, in wild-type mice.

3.1633 The transmembrane domain of podoplanin is required for its association with lipid rafts and the induction of epithelial-mesenchymal transition
Fernandez-Munoz, B., Yurrita, M.M., Martin-Villar, E., Carroasco-Ramirez, P., Megias, D., renart, J. and Quintanilla, M.
Int. J. Biochem. Cell Biol., ***43****, 886-896 (2011)*

Podoplanin is a transmembrane glycoprotein that is upregulated in cancer and was reported to induce an epithelial-mesenchymal transition (EMT) in MDCK cells. The promotion of EMT was dependent on podoplanin binding to ERM (ezrin, radixin, moesin) proteins through its cytoplasmic (CT) domain, which led to RhoA-associated kinase (ROCK)-dependent ERM phosphorylation. Using detergent-resistant membrane (DRM) assays, as well as transmembrane (TM) interactions and ganglioside GM1 binding, we present evidence supporting the localization of podoplanin in raft platforms important for cell signalling. Podoplanin mutant constructs harbouring a heterologous TM region or lacking the CT tail were unable to associate with DRMs, stimulate ERM phosphorylation and promote EMT or cell migration. Similar effects were observed upon disruption of a GXXXG motif within the TM domain, which is involved in podoplanin self-assembly. In contrast, deletion of the extracellular (EC) domain did not affect podoplanin DRM association. Together, these data suggest that both the CT and TM domains are required for podoplanin localization in raft platforms, and that this association appears to be necessary for podoplanin-mediated EMT and cell migration.

3.1634 Keratin 23, a novel DPC4/Smad4 target gene which binds 14-3-3ε

Liffers, S-T., Maghnouj, A., Munding, J.B., Jackstadt, R., Herbrand, U., Schulenborg, T., Marcus, K., Klein-Scory, S., Schmiegel, W., Schwarte-Waldhoff, I., Meyer, H.E., Stühler, K. and Hanh, S.A.
*BMC Cancer, **11:137**, (2011)*

Background
Inactivating mutations of SMAD4 are frequent in metastatic colorectal carcinomas. In previous analyses, we were able to show that restoration of Smad4 expression in Smad4-deficient SW480 human colon carcinoma cells was adequate to suppress tumorigenicity and invasive potential, whereas in vitro cell growth was not affected. Using this cellular model system, we searched for new Smad4 targets comparing nuclear subproteomes derived from Smad4 re-expressing and Smad4 negative SW480 cells.
Methods
High resolution two-dimensional (2D) gel electrophoresis was applied to identify novel Smad4 targets in the nuclear subproteome of Smad4 re-expressing SW480 cells. The identified candidate protein Keratin 23 was further characterized by tandem affinity purification. Immunoprecipitation, subfractionation and immunolocalization studies in combination with RNAi were used to validate the Keratin 23-14-3-3ε interaction.
Results
We identified keratins 8 and 18, heat shock proteins 60 and 70, plectin 1, as well as 14-3-3ε and γ as novel proteins present in the KRT23-interacting complex. Co-immunoprecipitation and subfractionation analyses as well as immunolocalization studies in our Smad4-SW480 model cells provided further evidence that KRT23 associates with 14-3-3ε and that Smad4 dependent KRT23 up-regulation induces a shift of the 14-3-3ε protein from a nuclear to a cytoplasmic localization.
Conclusion
Based on our findings we propose a new regulatory circuitry involving Smad4 dependent up-regulation of KRT23 (directly or indirectly) which in turn modulates the interaction between KRT23 and 14-3-3ε leading to a cytoplasmic sequestration of 14-3-3ε. This cytoplasmic KRT23-14-3-3 interaction may alter the functional status of the well described 14-3-3 scaffold protein, known to regulate key cellular processes, such as signal transduction, cell cycle control, and apoptosis and may thus be a previously unappreciated facet of the Smad4 tumor suppressive circuitry.

3.1635 BAX supports the mitochondrial network, promoting bioenergetics in nonapoptotic cells

Boohaker, R.J., Zhang, G., Carlson, A.L., Nemec, K.N. and Khaled, A.R.
*AM. J: Physiol. Cell. Physiol., **300**, C1466-C1478 (2011)*

The dual functionality of the tumor suppressor BAX is implied by the nonapoptotic functions of other members of the BCL-2 family. To explore this, mitochondrial metabolism was examined in BAX-deficient HCT-116 cells as well as primary hepatocytes from BAX-deficient mice. Although mitochondrial density and mitochondrial DNA content were the same in BAX-containing and BAX-deficient cells, MitoTracker staining patterns differed, suggesting the existence of BAX-dependent functional differences in mitochondrial physiology. Oxygen consumption and cellular ATP levels were reduced in BAX-deficient cells, while glycolysis was increased. These results suggested that cells lacking BAX have a deficiency in the ability to generate ATP through cellular respiration. This conclusion was supported by detection of reduced citrate synthase activity in BAX-deficient cells. In nonapoptotic cells, a portion of BAX associated with mitochondria and a sequestered, protease-resistant form was detected. Inhibition of BAX with small interfering RNAs reduced intracellular ATP content in BAX-containing cells. Expression of either full-length or COOH-terminal-truncated BAX in BAX-deficient cells rescued ATP synthesis and oxygen consumption and reduced glycolytic activity, suggesting that this metabolic function of BAX was not dependent upon its COOH-terminal helix. Expression of BCL-2 in BAX-containing cells resulted in a subsequent loss of ATP measured, implying that, even under nonapoptotic conditions, an antagonistic interaction exists between the two proteins. These findings infer that a basal amount of BAX is necessary to maintain energy production via aerobic respiration.

3.1636 Omeprazole Inhibits Proliferation and Modulates Autophagy in Pancreatic Cancer Cells

Udelnow, A., Kreyes, A., Ellinger, S., Landfester, K., Walther, P., Klapperstueck, T., Wohlrab, J., Henne-Bruns, D., Knippschild, U. and Würl, P.
*PloS One, **6(5)**, e20143 (2011)*

Background

Omeprazole has recently been described as a modulator of tumour chemoresistance, although its underlying molecular mechanisms remain controversial. Since pancreatic tumours are highly chemoresistant, a logical step would be to investigate the pharmacodynamic, morphological and biochemical effects of omeprazole on pancreatic cancer cell lines.
Methodology/Principal Findings
Dose-effect curves of omeprazole, pantoprazole, gemcitabine, 5-fluorouracil and the combinations of omeprazole and 5-fluorouracil or gemcitabine were generated for the pancreatic cancer cell lines MiaPaCa-2, ASPC-1, Colo357, PancTu-1, Panc1 and Panc89. They revealed that omeprazole inhibited proliferation at probably non-toxic concentrations and reversed the hormesis phenomena of 5-fluorouracil. Electron microscopy showed that omeprazole led to accumulation of phagophores and early autophagosomes in ASPC-1 and MiaPaCa-2 cells. Signal changes indicating inhibited proliferation and programmed cell death were found by proton NMR spectroscopy of both cell lines when treated with omeprazole which was identified intracellularly. Omeprazole modulates the lysosomal transport pathway as shown by Western blot analysis of the expression of LAMP-1, Cathepsin-D and β-COP in lysosome- and Golgi complex containing cell fractions. Acridine orange staining revealed that the pump function of the vATPase was not specifically inhibited by omeprazole. Gene expression of the autophagy-related LC3 gene as well as of Bad, Mdr-1, Atg12 and the vATPase was analysed after treatment of cells with 5-fluorouracil and omeprazole and confirmed the above mentioned results.
Conclusions
We hypothesise that omeprazole interacts with the regulatory functions of the vATPase without inhibiting its pump function. A modulation of the lysosomal transport pathway and autophagy is caused in pancreatic cancer cells leading to programmed cell death. This may circumvent common resistance mechanisms of pancreatic cancer. Since omeprazole use has already been established in clinical practice these results could lead to new clinical applications.

3.1637 Delayed Phosphorylation of Classical Protein Kinase C (PKC) Substrates Requires PKC Internalization and Formation of the Pericentrion in a Phospholipase D (PLD)-dependent Manner
El-Osta, M.A., Idkowiak-Baldys, J. and Hannun, Y.A.
*J. Biol. Chem., **286(22)**, 19340-19353 (2011)*

It was previously demonstrated that sustained activation (30–60 min) of protein kinase C (PKC) results in translocation of PKC α and βII to the pericentrion, a dynamic subset of the recycling compartment whose formation is dependent on PKC and phospholipase D (PLD). Here we investigated whether the formation of the pericentrion modulates the ability of PKC to phosphorylate substrates, especially if it reduces substrate phosphorylation by sequestering PKC. Surprisingly, using an antibody that detects phosphosubstrates of classical PKCs, the results showed that the majority of PKC phosphosubstrates are phosphorylated with delayed kinetics, correlating with the time frame of PKC translocation to the pericentrion. Substrate phosphorylation was blocked by PLD inhibitors and was not observed in response to activation of a PKC βII mutant (F663D) that is defective in interaction with PLD and in internalization. Phosphorylation was also inhibited by blocking clathrin-dependent endocytosis, demonstrating a requirement for endocytosis for the PKC-dependent major phosphorylation effects. Serotonin receptor activation by serotonin showed a similar response to phorbol 12-myristate 13-acetate, implicating a potential role of delayed kinetics in G protein-coupled receptor signaling. Evaluation of candidate substrates revealed that the phosphorylation of the PKC substrate p70S6K kinase behaved in a similar manner. Gradient-based fractionation revealed that the majority of these PKC substrates reside within the pericentrion-enriched fractions and not in the plasma membrane. Finally, proteomic analysis of the pericentrion-enriched fractions revealed several proteins as known PKC substrates and/or proteins involved in endocytic trafficking. These results reveal an important role for PKC internalization and for the pericentrion as key determinants/amplifiers of PKC action.

3.1638 Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage
Liu, L., Zhang, Z. and Xing, D.
*Free Radical Biology & Medicine, **51**, 53-68 (2011)*

Lysosomal photosensitizers have been used in photodynamic therapy. The combination of such photosensitizers and light causes lysosomal photodamage, inducing cell death. Lysosomal disruption can lead to apoptosis but its signaling pathways remain to be elucidated. In this study, *N*-aspartyl chlorin e6 (NPe6), an effective photosensitizer that preferentially accumulates in lysosomes, was used to study the mechanism of apoptosis caused by lysosomal photodamage. Apoptosis in living human lung adenocarcinoma cells (ASTC-a-1) after NPe6–photodynamic treatment (NPe6–PDT) was studied using

real-time single-cell analysis. Our results demonstrated that NPe6–PDT induced rapid generation of reactive oxygen species (ROS). The photodynamically produced ROS caused a rapid destruction of lysosomes, leading to release of cathepsins, and the ROS scavengers vitamin C and NAC prevent the effects. Then the following spatiotemporal sequence of cellular events was observed during cell apoptosis: Bcl-2-associated X protein (Bax) activation, cytochrome *c* release, and caspase-9/-3 activation. Importantly, the activation of Bax proved to be a crucial event in this apoptotic machinery, because suppressing the endogenous Bax using siRNA could significantly inhibit cytochrome *c* release and caspase-9/-3 activation and protect the cell from death. In conclusion, this study demonstrates that PDT with lysosomal photosensitizer induces Bax activation and subsequently initiates the mitochondrial apoptotic pathway.
Keywords: Lysosomal photodamage; NPe6–PDT; Bax; Apoptosis; Mitochondrial pathway; Free radicals
Abbreviations: AIF, apoptosis-inducing factor; Bax, Bcl-2-associated X protein; Bid, BH3-interacting-domain death agonist; CFP, cyan fluorescent protein; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; H_2DCFDA, dichlorodihydrofluorescein diacetate; LD_{50}, 50% lethal dose; LD_{90}, 90% lethal dose; NAC, *N*-acetylcysteine; NPe6, *N*-aspartyl chlorin e6; OMM, outer mitochondrial membrane; PBS, phosphate-buffered saline; PDT, photodynamic treatment; RFP, red fluorescent protein; RNAi, RNA interference; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential

3.1639 Conserved GXXXG- and S/T-Like Motifs in the Transmembrane Domains of NS4B Protein Are Required for Hepatitis C Virus Replication
Han, Q., Aligo, J., Manna, D., Belton, K., Chintapalli, S.V., Hong, Y., Patterson, R.L., van Rossum, D.B. and Konan, K.V.
J. Virol., ***85(13)****, 6464-6479 (2011)*

Hepatitis C virus (HCV) nonstructural protein 4B (NS4B) is an integral membrane protein, which plays an important role in the organization and function of the HCV replication complex (RC). Although much is understood about its amphipathic N-terminal and C-terminal domains, we know very little about the role of the transmembrane domains (TMDs) in NS4B function. We hypothesized that in addition to anchoring NS4B into host membranes, the TMDs are engaged in intra- and intermolecular interactions required for NS4B structure/function. To test this hypothesis, we have engineered a chimeric JFH1 genome containing the Con1 NS4B TMD region. The resulting virus titers were greatly reduced from those of JFH1, and further analysis indicated a defect in genome replication. We have mapped this incompatibility to NS4B TMD1 and TMD2 sequences, and we have defined putative TMD dimerization motifs (GXXXG in TMD2 and TMD3; the S/T cluster in TMD1) as key structural/functional determinants. Mutations in each of the putative motifs led to significant decreases in JFH1 replication. Like most of the NS4B chimeras, mutant proteins had no negative impact on NS4B membrane association. However, some mutations led to disruption of NS4B foci, implying that the TMDs play a role in HCV RC formation. Further examination indicated that the loss of NS4B foci correlates with the destabilization of NS4B protein. Finally, we have identified an adaptive mutation in the NS4B TMD2 sequence that has compensatory effects on JFH1 chimera replication. Taken together, these data underscore the functional importance of NS4B TMDs in the HCV life cycle.

3.1640 Cholesterol sequestration by nystatin enhances the uptake and activity of endostatin in endothelium via regulating distinct endocytic pathways
Chen, Y., Wang, S., Lu, X., Zhang, H., Fu, Y. and Luo, Y.
Blood, ***117****, 6392-6403 (2011)*

Specific internalization of endostatin into endothelial cells has been proved to be important for its biologic functions. However, the mechanism of endostatin internalization still remains elusive. In this study, we report for the first time that both caveolae/lipid rafts and clathrin-coated pits are involved in endostatin internalization. Inhibition of either the caveolae pathway or the clathrin pathway with the use of chemical inhibitors, small interfering RNAs, or dominant-negative mutants alters endostatin internalization in vitro. Intriguingly, cholesterol sequestration by nystatin, a polyene antifungal drug, significantly enhances endostatin uptake by endothelial cells through switching endostatin internalization predominantly to the clathrin-mediated pathway. Nystatin-enhanced internalization of endostatin also increases its inhibitory effects on endothelial cell tube formation and migration. More importantly, combined treatment with nystatin and endostatin selectively enhances endostatin uptake and biodistribution in tumor blood vessels and tumor tissues but not in normal tissues of tumor-bearing mice, ultimately resulting in elevated antiangiogenic and antitumor efficacies of endostatin in vivo. Taken together, our data show a novel

mechanism of endostatin internalization and support the potential application of enhancing the uptake and therapeutic efficacy of endostatin via regulating distinct endocytic pathways with cholesterol-sequestering agents.

3.1641 Caveolin-1 and force regulation in porcine airway smooth muscle

Satish, V., Yang, B., Meuchel, L.W., VanOosten, S.K., Ryu, A.J., Thompson, M.A., Prakash, Y.S. and Pabelick, C.

*Am. J. Physiol. Lung Cell. Mol. Physiol., **300**, L920-L929 (2011)*

Caveolae are specialized membrane microdomains expressing the scaffolding protein caveolin-1. We recently demonstrated the presence of caveolae in human airway smooth muscle (ASM) and the contribution of caveolin-1 to intracellular calcium ($[Ca^{2+}]_i$) regulation. In the present study, we tested the hypothesis that caveolin-1 regulates ASM contractility. We examined the role of caveolins in force regulation of porcine ASM under control conditions as well as TNF-α-induced airway inflammation. In porcine ASM strips, exposure to 10 mM methyl-β-cyclodextrin (CD) or 5 μM of the caveolin-1 specific scaffolding domain inhibitor peptide (CSD) resulted in time-dependent decrease in force responses to 1 μM ACh. Overnight exposure to the cytokine TNF-α (50 ng/ml) accelerated and increased caveolin-1 expression and enhanced force responses to ACh. Suppression of caveolin-1 with small interfering RNA mimicked the effects of CD or CSD. Regarding mechanisms by which caveolae contribute to contractile changes, inhibition of MAP kinase with 10 μM PD98059 did not alter control or TNF-α-induced increases in force responses to ACh. However, inhibiting RhoA with 100 μM fasudil or 10 μM Y27632 resulted in significant decreases in force responses, with lesser effects in TNF-α exposed samples. Furthermore, Ca^{2+} sensitivity for force generation was substantially reduced by fasudil or Y27632, an effect even more enhanced in the absence of caveolin-1 signaling. Overall, these results indicate that caveolin-1 is a critical player in enhanced ASM contractility with airway inflammation.

3.1642 Cholesterol Regulates μ-Opioid Receptor-Induced β-Arrestin 2 Translocation to Membrane Lipid Rafts

Qiu, Y., Wang, Y., Law, P-Y., Chen, H-Z. and Loh, H.H.

*Mol. Pharmacol., **80(1)**, 210-218 (2011)*

μ-Opioid receptor (OPRM1) is mainly localized in lipid raft microdomains but internalizes through clathrin-dependent pathways. Our previous studies demonstrated that disruption of lipid rafts by cholesterol-depletion reagent blocked the agonist-induced internalization of OPRM1 and G protein-dependent signaling. The present study demonstrated that reduction of cholesterol level decreased and culturing cells in excess cholesterol increased the agonist-induced internalization and desensitization of OPRM1, respectively. Further analyses indicated that modulation of cellular cholesterol level did not affect agonist-induced receptor phosphorylation but did affect membrane translocation of β-arrestins. The translocation of β-arrestins was blocked by cholesterol reduction, and the effect could be reversed by incubating with cholesterol. OptiPrep gradient separation of lipid rafts revealed that excess cholesterol retained more receptors in lipid raft domains and facilitated the recruitment of β-arrestins to these microdomains upon agonist activation. Moreover, excess cholesterol could evoke receptor internalization and protein kinase C-independent extracellular signal-regulated kinases activation upon morphine treatment. Therefore, these results suggest that cholesterol not only can influence OPRM1 localization in lipid rafts but also can effectively enhance the recruitment of β-arrestins and thereby affect the agonist-induced trafficking and agonist-dependent signaling of OPRM1.

3.1643 ST101 induces a novel 17kDa APP cleavage that precludes Aβ generation in vivo

Green, K.N., Khashwji, K., Estrada, T. and LaFerla, F.M.

*Ann. Neurol., **69(5)**, 831-844 (2011)*

Objective:
Inhibiting Aβ generation is a prime therapeutic goal for preventing or treating Alzheimer disease. Here we sought to identify any disease-modifying properties of an azaindolizinone derivative, spiro[imidazo[1,2-a]pyridine-3,2-idan]-2(3H)-one (ST101 or ZSET1446).
Methods:
The effects of ST101 were studied in 3xTg-AD mice and young cynomolgus monkeys using a combination of biochemical and histological analyses.
Results:

Here we describe that ST101 induces cleavage of APP protein at a novel site, generating a 17kDa C-terminal fragment. This 17kDa APP cleavage product does not appear to be a substrate for either α- or β-secretase, and thus bypasses generation of Aβ. ST101 is orally active, efficacious at low doses, improves memory function, and robustly reduces brain Aβ in transgenic mice and nonhuman primates.
Interpretation:
Using rodent and nonhuman primate models, we show that ST101 represents a novel class of small molecules that reduce central nervous system levels of Aβ by inducing an alternate pathway of APP cleavage.

3.1644 Heme oxygenase-1 expression protects melanocytes from stress-induced cell death: implications for vitiligo

Elassiuty, Y.E., Klarquist, J., Speiser, J., Yousef, R.M., El Refaee, A.A., Hunter, N.S., Shaker, O.G., Gundeti, M., Nieuweboer-Krobotova, L. and Le Poole, I.C.
Exp. Dermatol., ***20(6)****, 496-501 (2011)*

To study protection of melanocytes from stress-induced cell death by heme oxygenases during depigmentation and repigmentation in vitiligo, expression of isoforms 1 and 2 was studied in cultured control and patient melanocytes and normal skin explants exposed to UV or bleaching agent 4-TBP. Similarly, expression of heme oxygenases was followed in skin from vitiligo patients before and after PUVA treatment. Single and double immunostainings were used in combination with light and confocal microscopic analysis and Western blotting. Melanocyte expression of heme oxygenase 1 is upregulated, whereas heme oxygenase 2 is reduced in response to UV and 4-TBP. Upregulation of inducible heme oxygenase 1 was also observed in UV-treated explant cultures, in skin of successfully PUVA-treated patients and in melanocytes cultured from vitiligo non-lesional skin. Heme oxygenase encoding genes were subsequently cloned to study consequences of either gene product on cell viability, demonstrating that HO-1 but not HO-2 overexpression offers protection from stress-induced cell death in MTT assays. HO-1 expression by melanocytes may contribute to beneficial effects of UV treatment for vitiligo patients.

3.1645 Interferon-stimulated gene *ISG12b2* is localized to the inner mitochondrial membrane and mediates virus-induced cell death

Lu, M-Y. and Liao, F.
Cell Death and Differentiation, ***18(6)****, 925-936 (2011)*

Interferons (IFNs) are crucial for host defence against viruses. Many IFN-stimulated genes (ISGs) induced by viral infection exert antiviral effects. Microarray analysis of gene expression induced in liver tissues of mice on dengue virus (DENV) infection has led to identification of the ISG gene *ISG12b2*. *ISG12b2* is also dramatically induced on DENV infection of Hepa 1-6 cells (mouse hepatoma cell line). Here, we performed biochemical and functional analyses of ISG12b2. We demonstrate that ISG12b2 is an inner mitochondrial membrane (IMM) protein containing a cleavable mitochondrial targeting sequence and multiple transmembrane segments. Overexpression of ISG12b2 in Hepa 1-6 induced release of cytochrome *c* from mitochondria, disruption of the mitochondrial membrane potential, and activation of caspase-9, caspase-3, and caspase-8. Treatment of ISG12b2-overexpressing Hepa 1-6 with inhibitors of pan-caspase, caspase-9, or caspase-3, but not caspase-8, reduced apoptotic cell death, suggesting that ISG12b2 activates the intrinsic apoptotic pathway. Of particular interest, we further demonstrated that ISG12b2 formed oligomers, and that ISG12b2 was able to mediate apoptosis through both Bax/Bak-dependent and Bax/Bak-independent pathways. Our study demonstrates that the ISG12b2 is a novel IMM protein induced by IFNs and regulates mitochondria-mediated apoptosis during viral infection.

3.1646 Continuous Density Gradients to Study Argonaute and GW182 Complexes Associated with the Endocytic Pathway

Gibbings, D.
Methods in Mol. Biol., ***725****, 63-76 (2011)*

Most complexes involved in RNA silencing were thought to be concentrated in cytoplasmic sites called P-bodies in the absence of stress. Accumulating evidence suggests that distinct cellular organelles or sites may be involved in the maturation of RNA-induced silencing complexes (RISC), decapping and deadenylation of miRNA-repressed mRNA, transport of translationally repressed mRNA, and disassembly of RISC complexes. Significant fractions of proteins essential for RNA silencing associate with membranes

in general (GW182, AGO, and DICER), or more specifically with endoplasmic reticulum and Golgi (AGO), or endosomes and multivesicular bodies (AGO, GW182). In contrast, mRNA decapping and decay occur mainly in the cytoplasm. Continuous density gradients capable of partitioning these cellular compartments are valuable tools in efforts to decipher the complexes, trafficking and regulation of RISC throughout its biogenesis, action and turnover.

3.1647 Partial colocalization of retinoschisin (RS1) and the Na+/K+- ATPase subunit in membrane rafts and their role in signaling

Härtinger, T., Walczak, Y., Weber, B.H.F.and Langmann, T.

*Medizinische Genetik, **23(1)**, P-basic-162 (2011)*

Purpose
Previous studies have shown that Retinoschisin (RS1) binds to the Na+/K+-ATPase within the retina. In other cell types like cardiac myocytes, the Na+/K+-ATPase is located in detergent resistant membranes (DRMs), which represent rafts floating on the cell surface. These membrane
membrane domains can serve as platforms for receptor-ligand interactions and intracellular signaling. We hypothesized that RS1 binding to the β2-subunit of the α3β2 Na+/K+-ATPase occurs in retinal membrane rafts and thereby may regulate signaling functions.
Methods
To isolate DRMs from WERI-RB1 cells and mouse retinas, cells were pelleted and lysed after treatment with 2% Lubrol or Triton-X 100 in TNE buffer, respectively at 4°C. A discontinuous density gradient centrifugation was then performed with OptiPrep® solution. After 4 hours of centrifugation at 100.000x g, six fractions were collected from top to bottom. The proteins were precipitated using methanol/chloroform before resuspension in buffer containing 1% SDS. The samples were then analyzed by Western Blot using antibodies against the β2 subunit of the Na+/K+-ATPase, RS1 and flotillin as a DRM-marker. For signaling approaches retinal lysates or cryo-sections were stained with antibodies against phospho-ERK1/2 and p38 MAPK.
Results
We could successfully implement a raft isolation procedure from WERI cells and mouse retinas. DRMs were floating up in the low density fractions as demonstrated by strong staining with the marker flotillin. Staining for the β2 subunit of the Na+/K+-ATPase also showed high signals in raft fractions from WERI-RB1 cells. In retinal samples, staining for the β2 subunit of the Na+/K+-ATPase was strong in the raft fraction, but was also present in high density fractions. We could further show that RS1 was also partially present in DRMs of WERI cells and wild-type retinas. Our results also suggest that ERK1/2 and p38 MAPK are activated in Retinoschisin-deficient and ATP1b2 knock out mice at postnatal days 14 and 10, respectively.
Conclusions
Our experiments demonstrate that the α3β2 subunits of Na+/K+- ATPase and RS1 partially colocalize in retinal rafts. This distribution of RS1 implicates that it has a dual function. On the one hand, RS1 may serve as a secreted adhesion molecule and on the other hand it may trigger intracellular signaling by interaction with the Na+/K+-ATPase in rafts as signaling platforms.

3.1648 Rituximab Targets Podocytes in Recurrent Focal Segmental Glomerulosclerosis

Fornoni, A. et al

*Science Translational Medicine, **3(85)**, 85ra46 (2011)*

Focal segmental glomerulosclerosis (FSGS) is a glomerular disease characterized by proteinuria, progression to end-stage renal disease, and recurrence of proteinuria after kidney transplantation in about one-third of patients. It has been suggested that rituximab might treat recurrent FSGS through an unknown mechanism. Rituximab not only recognizes CD20 on B lymphocytes, but might also bind sphingomyelin phosphodiesterase acid-like 3b (SMPDL-3b) protein and regulate acid sphingomyelinase (ASMase) activity. We hypothesized that rituximab prevents recurrent FSGS and preserves podocyte SMPDL-3b expression. We studied 41 patients at high risk for recurrent FSGS, 27 of whom were treated with rituximab at time of kidney transplant. SMPDL-3b protein, ASMase activity, and cytoskeleton remodeling were studied in cultured normal human podocytes that had been exposed to patient sera with or without rituximab. Rituximab treatment was associated with lower incidence of posttransplant proteinuria and stabilization of glomerular filtration rate. The number of SMPDL-3b$^+$ podocytes in postreperfusion biopsies was reduced in patients who developed recurrent FSGS. Rituximab partially prevented SMPDL-3b and ASMase down-regulation that was observed in podocytes treated with the sera of patients with

recurrent FSGS. Overexpression of SMPDL-3b or treatment with rituximab was able to prevent disruption of the actin cytoskeleton and podocyte apoptosis induced by patient sera. This effect was diminished in cultured podocytes where *SMPDL-3b* was silenced. Our study suggests that treatment of high-risk patients with rituximab at time of kidney transplant might prevent recurrent FSGS by modulating podocyte function in an SMPDL-3b–dependent manner.

3.1649 DNA Nuclear Targeting Sequences for Non-Viral Gene Delivery

Van Gaal, E.V.B., Oosting, R., van Eijk, R., Bakowska, M., Feyen, D., Kok, R.J., Hennink, W.E., Crommelin, D.J.A. and Mastrobattista, E.
Pharm. Res., ***28(7)***, *1707-1722 (2011)*

Purpose
To evaluate if introduction of DNA nuclear Targeting Sequences (DTS; i.e. recognition sequences for endogenous DNA-binding proteins) in plasmid DNA (pDNA) leads to increased transfection efficiency of non-viral gene delivery by virtue of enhanced nuclear import of the pDNA.
Methods
A set of DTS was identified and cloned into EGFP-reporter plasmids controlled by the CMV-promoter. These pDNA constructs were delivered into A431 and HeLa cells using standard electroporation, pEI-based polyfection or lipofection methods. The amount of pDNA delivered into the nucleus was determined by qPCR; transfection efficiency was determined by flow cytometry.
Results
Neither of these DTS increased transgene expression. We varied several parameters (mitotic activity, applied dose and delivery strategy), but without effect. Although upregulated transgene expression was observed after stimulation with TNF-α, this effect could be ascribed to non-specific upregulation of transcription rather than enhanced nuclear import. Nuclear copy numbers of plasmids containing or lacking a DTS did not differ significantly after lipofectamine-based transfection in dividing and non-dividing cells.
Conclusion
No beneficial effects of DTS on gene expression or nuclear uptake were observed in this study.

3.1650 ER-stress-inducible Herp, facilitates the degradation of immature nicastrin

Marutani, T., Maeda, T., Tanabe, C., Zou, K., Araki, W., Kokame, K., Michikawa, M. and Komano, H.
Biochim. Biophys. Acta, ***1810****, 790-798 (2011)*

Background
Herp is an endoplasmic reticulum (ER)-stress-inducible membrane protein harboring an ubiquitin-like domain (ULD). However, its biological functions are not fully understood. Here, we examined the role of Herp in the degradation of γ-secretase components.
Methods
Effects of ULD-lacking Herp (ΔUb-Herp) expression on the degradation of γ-secretase components were analyzed.
Results
The cellular expression of ΔUb-Herp was found to inhibit the degradation of overexpressed immature nicastrin and full-length presenilin. The mechanisms underlying Herp-mediated nicastrin degradation was further analyzed. We found that immature nicastrin accumulates in the ER of ΔUb-Herp overexpressing cells or Herp-deficient cells more than that in the ER of wild-type cells. Further, ΔUb-Herp expression inhibited nicastrin ubiquitination, suggesting that the ULD of Herp is likely involved in nicastrin ubiquitination. Co-immunoprecipitation study showed that Herp as well as ΔUb-Herp potentially interacts with nicastrin, mediating nicastrin interaction with p97, which functions in retranslocation of misfolded proteins from the ER to the cytosol.
Conclusions
Thus, Herp is likely involved in degradation of immature nicastrin by facilitating p97-dependent nicastrin retranslocation and ubiquitination. General significance: We suggest that Herp could play a role in the elimination of the excess unassembled components of a multimeric complex.
Research highlights
► The deletion of the ubiquitin-like domain of Herp impairs the degradation of nicastrin. ► Herp facilitates the ubiquitination of nicastrin. ► Herp plays a role for the elimination of the excess unassembled components of a multimeric complex.

3.1651 **Phr1p, a glycosylphosphatidylinsitol-anchored β(1,3)-glucanosyltransferase critical for hyphal wall formation, localizes to the apical growth sites and septa in *Candida albicans***
Ragni, E., Calderon, J., Fascio, U., Sipiczki, M., Fonzi, W. and Popolo, L
Fungal Genetics and Biology, ***48***, *793-805 (2011)*

Cell wall biogenesis is a dynamic process relying on the coordinated activity of several extracellular enzymes. *PHR1* is a pH-regulated gene of *Candida albicans* encoding a glycosylphosphatidylinositol-anchored β(1,3)-glucanosyltransferase of family GH72 which acts as a cell wall remodelling enzyme and is crucial for morphogenesis and virulence. In order to explore the function of Phr1p, we obtained a green fluorescent protein (GFP) fusion to determine its localization. During induction of vegetative growth, Phr1p-GFP was concentrated in the plasma membrane of the growing bud, in the mother-bud neck, and in the septum. Phr1p-GFP was recovered in the detergent-resistant membranes indicating its association with the lipid rafts as the wild type Phr1p. Upon induction of hyphal growth, Phr1p-GFP highly concentrated at the apex of the germ tubes and progressively distributed along the lateral sides of the hyphae. Phr1p-GFP also labelled the hyphal septa, where it colocalized with chitin. Localization to the hyphal septa was perturbed in nocodazole-treated cells, whereas inhibition of actin polymerization hindered the apical localization. Electron Microscopy analysis of the hyphal wall ultrastructure of a *PHR1* null mutant showed loss of compactness and irregular organization of the surface layer. These observations indicate that Phr1p plays a crucial role in hyphal wall formation, a highly regulated process on which morphogenesis and virulence rely.

3.1652 **Maturation of BRI2 generates a specific inhibitor that reduces APP processing at the plasma membrane and in endocytic vesicles**
Matsuda, S., Matsuda, Y., Snapp, E.L. and D'Adamio, L.
Neurobiology of Aging, ***32***, *1400-1408 (2011)*

Processing of the amyloid-β (Aβ) precursor protein (*APP*) has been extensively studied since it leads to production of Aβ peptides. Toxic forms of Aβ aggregates are considered the cause of Alzheimer's disease (AD). On the other end, BRI2 is implicated in APP processing and Aβ production. We have investigated the precise mechanism by which BRI2 modulates APP cleavages and have found that BRI2 forms a mature BRI2 polypeptide that is transported to the plasma membrane and endosomes where it interacts with mature APP. Notably, immature forms of APP and BRI2 fail to interact. Mature BRI2 inhibits APP processing by α-, β- and γ-secretases on the plasma membrane and in endocytic compartments. Thus, BRI2 is a specific inhibitor that reduces secretases' access to APP in the intracellular compartments where APP is normally processed.

3.1653 **Effect of Glycans and the Glycophosphatidylinositol Anchor on Strain Dependent Conformations of Scrapie Prion Protein: Improved Purifications and Infrared Spectra**
Baron, G.S., Hughson, A.G., Raymond, G.J., Offerdahl, D.K., Barton, K.A., Raymond, L.D., Dorward, D.W. and Caughey, B.
Biochemistry, ***50***, *4479-4490 (2011)*

Mammalian prion diseases involve conversion of normal prion protein, PrP^{C}, to a pathological aggregated state (PrP^{res}). The three-dimensional structure of PrP^{res} is not known, but infrared (IR) spectroscopy has indicated high, strain-dependent β-sheet content. PrP^{res} molecules usually contain a glycophosphatidylinositol (GPI) anchor and large Asn-linked glycans, which can also vary with strain. Using IR spectroscopy, we tested the conformational effects of these post-translational modifications by comparing wild-type PrP^{res} with GPI- and glycan-deficient PrP^{res} produced in GPI-anchorless PrP transgenic mice. These analyses required the development of substantially improved purification protocols. Spectra of both types of PrP^{res} revealed conformational differences between the 22L, ME7, and Chandler (RML) murine scrapie strains, most notably in bands attributed to β-sheets. These PrP^{res} spectra were also distinct from those of the hamster 263K scrapie strain. Spectra of wild-type and anchorless 22L PrP^{res} were nearly indistinguishable. With ME7 PrP^{res}, modest differences between the wild-type and anchorless spectra were detected, notably an ~2 cm^{-1} shift in an apparent β-sheet band. Collectively, the data provide evidence that the glycans and anchor do not grossly affect the strain-specific secondary structures of PrP^{res}, at least relative to the differences observed between strains, but can subtly affect turns and certain β-sheet components. Recently reported H–D exchange analyses of anchorless PrP^{res} preparations strongly suggested the presence of strain-dependent, solvent-inaccessible β-core structures throughout most of the C-terminal half of PrP^{res} molecules, with no remaining α-helix. Our IR data provide evidence that similar core structures also comprise wild-type PrP^{res}.

3.1654 Enzymatic Properties and Regulation of the Native Isozymes of Retinal Membrane Guanylyl Cyclase (RetGC) from Mouse Photoreceptors

Peshenko, I.V., Olshevskaya, E.V., Savchenko, A.B., Kara, S., Palczewski, K., Baehr, W. and Dizhoor, A.M.

*Biochemistry, **50**, 5590-5600 (2011)*

Mouse photoreceptor function and survival critically depend on Ca^{2+}-regulated retinal membrane guanylyl cyclase (RetGC), comprised of two isozymes, RetGC1 and RetGC2. We characterized the content, catalytic constants, and regulation of native RetGC1 and RetGC2 isozymes using mice lacking guanylyl cyclase activating proteins GCAP1 and GCAP2 and deficient for either *GUCY2F* or *GUCY2E* genes, respectively. We found that the characteristics of both native RetGC isozymes were considerably different from other reported estimates made for mammalian RetGCs: the content of RetGC1 per mouse rod outer segments (ROS) was at least 3-fold lower, the molar ratio (RetGC2:RetGC1) 6-fold higher, and the catalytic constants of both GCAP-activated isozymes between 12- and 19-fold higher than previously measured in bovine ROS. The native RetGC isozymes had different basal activity and were accelerated 5–28-fold at physiological concentrations of GCAPs. RetGC2 alone was capable of contributing as much as 135–165 μM cGMP s^{-1} or almost 23–28% to the maximal cGMP synthesis rate in mouse ROS. At the maximal level of activation by GCAP, this isozyme alone could provide a significantly high rate of cGMP synthesis compared to what is expected for normal recovery of a mouse rod, and this can help explain some of the unresolved paradoxes of rod physiology. GCAP-activated native RetGC1 and RetGC2 were less sensitive to inhibition by Ca^{2+} in the presence of GCAP1 (EC_{50Ca} ~132–139 nM) than GCAP2 (EC_{50Ca} ~50–59 nM), thus arguing that Ca^{2+} sensor properties of GCAP in a functional RetGC/GCAP complex are defined not by a particular target isozyme but the intrinsic properties of GCAPs themselves.

3.1655 KLP6: a newly identified kinesin that regulates the morphology and transport of mitochondria in neuronal cells

Tanaka, K., Sugiura, Y., Ichishita, R., Mihara, K. and Oka, T.

*J. Cell Sci., **124(14)**, 2457-2465 (2011)*

Mitochondria utilize diverse cytoskeleton-based mechanisms to control their functions and morphology. Here, we report a role for kinesin-like protein KLP6, a newly identified member of the kinesin family, in mitochondrial morphology and dynamics. An RNA interference screen using *Caenorhabditis elegans* led us to identify a *C. elegans* KLP-6 involved in maintaining mitochondrial morphology. We cloned a cDNA coding for a rat homolog of *C. elegans* KLP-6, which is an uncharacterized kinesin in vertebrates. A rat KLP6 mutant protein lacking the motor domain induced changes in mitochondrial morphology and significantly decreased mitochondrial motility in HeLa cells, but did not affect the morphology of other organelles. In addition, the KLP6 mutant inhibited transport of mitochondria during anterograde movement in differentiated neuro 2a cells. To date, two kinesins, KIF1Bα and kinesin heavy chain (KHC; also known as KIF5) have been shown to be involved in the distribution of mitochondria in neurons. Expression of the kinesin heavy chain/KIF5 mutant prevented mitochondria from entering into neurites, whereas both the KLP6 and KIF1Bα mutants decreased mitochondrial transport in axonal neurites. Furthermore, both KLP6 and KIF1Bα bind to KBP, a KIF1-binding protein required for axonal outgrowth and mitochondrial distribution. Thus, KLP6 is a newly identified kinesin family member that regulates mitochondrial morphology and transport.

3.1656 Aldolase directly interacts with ARNO and modulates cell morphology and acidic vesicle distribution

Merkulova, M., Hurtado-Lorenzo, A., Hosokawa, H., Zhuang, Z., Brown, D., Ausiello, D.A. and Marshansky, V.

*Am. J. Physiol. Cell Physiol., **300**, C1442-C1455 (2011)*

Previously, we demonstrated that the vacuolar-type H^+-ATPase (V-ATPase) a2-subunit functions as an endosomal pH sensor that interacts with the ADP-ribosylation factor (Arf) guanine nucleotide exchange factor, ARNO. In the present study, we showed that ARNO directly interacts not only with the a2-subunit but with all a-isoforms (a1–a4) of the V-ATPase, indicating a widespread regulatory interaction between V-ATPase and Arf GTPases. We then extended our search for other ARNO effectors that may modulate V-ATPase-dependent vesicular trafficking events and actin cytoskeleton remodeling. Pull-down experiments using cytosol of mouse proximal tubule cells (MTCs) showed that ARNO interacts with aldolase, but not with other enzymes of the glycolytic pathway. Direct interaction of aldolase with the

pleckstrin homology domain of ARNO was revealed by pull-down assays using recombinant proteins, and surface plasmon resonance revealed their high avidity interaction with a dissociation constant: $K_D = 2.84 \times 10^{-10}$ M. MTC cell fractionation revealed that aldolase is also associated with membranes of early endosomes. Functionally, aldolase knockdown in HeLa cells produced striking morphological changes accompanied by long filamentous cell protrusions and acidic vesicle redistribution. However, the 50% knockdown we achieved did not modulate the acidification capacity of endosomal/lysosomal compartments. Finally, a combination of small interfering RNA knockdown and overexpression revealed that the expression of aldolase is inversely correlated with gelsolin levels in HeLa cells. In summary, we have shown that aldolase forms a complex with ARNO/Arf6 and the V-ATPase and that it may contribute to remodeling of the actin cytoskeleton and/or the trafficking and redistribution of V-ATPase-dependent acidic compartments via a combination of protein-protein interaction and gene expression mechanisms.

3.1657 Subcellular Fractionation of Brain Tissue Using Free-Flow Electrophoresis
Islinger, M., Kirsch, J., Angermüller, S., Rotaru, R., Abdolzade-Bavil, A. and Weber, G.
*Nueromethods, **57(2)**, 27-45 (2011)*

Accurate annotation of protein identifications in organellar proteomics highly depends on the sample quality with special respect to contaminations from other subcellular compartments. In this respect, Freeflow
electrophoresis (FFE) offers a valuable alternative to classical centrifugation techniques, since it relies on quite different physical parameters. During the last years, FFE has been successfully used for the separation
of various organelles from different tissues, yet is largely unknown in the field of neurobiology.
Here we present two separation schemes for the fractionation of a synaptic preparation from rat brain using different modes of FFE. Isotachophoresis (ITP), a focusing technique separating organelles according
to their electrophoretic mobilities, was able to distribute the synaptosome sample into different subfractions:
mitochondrial cross contaminations showed the highest electrophoretic mobility and migrated nearest to the anode of the FFE instrument; proximate to these, proteins of the presynaptic compartment accumulated, whereas nearest to the cathode of the instrument postsynaptic marker proteins were predominantly
found. As a nonfocusing technique, zonal FFE does not possess a separation capacity comparable to ITP; however, due to a continuous separation mode, it is adapted to process higher sample amounts and can be used for large-scale separations. We applied zonal FFE to the same starting material as in ITP and were able to separate mitochondria from synaptic material of the preparation, thus offering a fast alternative to clean synaptosome preparations from residual mitochondrial contaminations.

3.1658 Methods to Monitor Cell Surface Expression and Endocytic Trafficking of CFTR in Polarized Epithelial Cells
Bomberger, J.M., Guggino, W.B. and Stanton, B.A.
*Methods in Mol. Biol., **741(3)**, 271-283 (2011)*

Cystic fibrosis transmembrane conductance regulator (CFTR)-mediated chloride secretion is critical to maintaining airway surface hydration and efficient mucociliary clearance in the upper airways. Mutations in CFTR in cystic fibrosis lead to reduced expression of functional CFTR channels at the apical plasma membrane of the airway epithelium, leading to dehydration of the airway surface liquid and diminished mucociliary clearance. Cell surface CFTR is modulated by changes in CFTR endocytosis and recycling, effectively altering the cell surface abundance of the channel. This chapter examines current methods employed to measure the cell surface expression of CFTR, as well as methods to monitor CFTR movement through the endocytic pathway.

3.1659 Chemical chaperone therapy: chaperone effect on mutant enzyme and cellular pathophysiology in β-galactosidase deficiency
Higaki, K. et al
*Hum. Mutat, **32**, 843-852 (2011)*

β-Galactosidase deficiency is a group of lysosomal lipid storage disorders with an autosomal recessive trait. It causes two clinically different diseases, G_{M1}-gangliosidosis and Morquio B disease. It is caused by heterogeneous mutations in the GLB1 gene coding for the lysosomal acid β-galactosidase. We have

previously reported the chaperone effect of N-octyl-4-epi-β-valienamine (NOEV) on mutant β-galactosidase proteins. In this study, we performed genotype analyses of patients with β-galactosidase deficiency and identified 46 mutation alleles including 9 novel mutations. We then examined the NOEV effect on mutant β-galactosidase proteins by using six strains of patient-derived skin fibroblast. We also performed mutagenesis to identify β-galactosidase mutants that were responsive to NOEV and found that 22 out of 94 mutants were responsive. Computational structural analysis revealed the mode of interaction between human β-galactosidase and NOEV. Moreover, we confirmed that NOEV reduced G_{M1} accumulation and ameliorated the impairments of lipid trafficking and protein degradation in β-galactosidase deficient cells. These results provided further evidence to NOEV as a promising chaperone compound for β-galactosidase deficiency

3.1660 Proteomic analysis of microvesicles derived from human colorectal cancer ascites
Choi, D-S. et al
*Proteomics, **11(13)**, 2745-2751 (2011)*

The presence of malignant ascites in the peritoneal cavity is a poor prognostic indicator of low survival rate. Various cancer cells, including those of colorectal cancer (CRC), release microvesicles (exosomes) into surrounding tissues and peripheral circulation including malignant ascites. Although recent progress has revealed that microvesicles play multiple roles in tumor progression, the protein composition and the pathological function of malignant ascites-derived microvesicles are still unknown. Here, we report the first global proteomic analyses of highly purified microvesicles derived from human CRC ascites. With 1-D SDS-PAGE and nano-LC-MS/MS analyses, we identified a total of 846 microvesicular proteins from ascites of three CRC patients with high confidence; 384 proteins were identified in at least two patients. We identified proteins that might function in tumor progression via disruption of epithelial polarity, migration, invasion, tumor growth, immune modulation, and angiogenesis. Furthermore, we identified several potential diagnostic markers of CRC including colon-specific surface antigens. Our proteomic analyses will help to elucidate diverse functions of microvesicles in cancer progression and will aid in the development of novel diagnostic tools for CRC.

3.1661 Dietary Fatty Acid in Rat Intestinal Cytosol is Associated With Caveolae
Siddiqi, S. and Mansbach, C.M.
*Gastroenterology, **140(5)**, Suppl. 1, S-452 (2011)*

The intestinal absorptive cell has no control over the amount of fatty acid (FA) presented to it. In humans, intestinal luminal FA concentrations after a fatty meal are 28 mM. We show here that rat proximal intestinal sacs incubated with 1 mM oleate have 1.6 μ mol FA/ mg cytosolic protein whereas it has been shown that after a fat meal, rats have only 10 and 2 fmol FA/mg cytosolic protein bound to liver (LFABP) and intestinal fatty acid binding proteins (IFABP). This raises the question of another binding mechanism for FA in cytosol to prevent membrane disruption by FA. Caveolin-1 containing lipid rafts, caveolae, present an alternative FA binding mechanism. Caveolae occupy 50% of the microvillus membrane presenting a large potential absorptive area. Methods: Intestinal sacs from rat proximal . intestine were incubated (370C) with TriacinC for 15 min to inhibit triglyceride synthesis, 1 mM 3H-oleate was added for 2 min and the sac placed on ice. Cytosol was isolated from sac enterocytes and chromatographed over a Sephacryl S-100 HR column or placed under an OptiPrep gradient. Results: Only 5% of the 3H-oleate but 60% of the protein was recovered from the Sepharose column with PBS as eluent whereas 95% of the 3H-oleate was recovered with 1% Triton X-100 as eluent. All the dpm were in the early eluting fractions suggesting the 3H-oleate was associated with a detergent resistant membrane (DRM) rather than FABP. On the OptiPrep gradient, 57% of the dpm were in the light fractions confirming a DRM location of oleate. The light fraction contained Caveolin-1,-2,-3, CD36, and intestinal alkaline phosphatase (IAP) but not L or IFABP, rab11, or fatty acid transport protein 4 (FATP4) by immunoblot. Immunodepletion of Caveolin-1 removed 90% of the 3H-oleate from the cytosol whereas CD36 and IAP immunodepletion removed 50%. Caveolin-2,-3, IgG, and clathrin immunodepletion removed only 20%. Immuno-precipitation (IP) of Caveolin-1 co-precipitated CD36 and IAP. IP of CD36 co-precipitated Caveolin-1 and IAP; IP of IAP precipitated CD36 and Caveolin-1. Electron microscopy of cytosol showed multiple 18.9 • }2.9 nm vesicles that increased to 33.4 • }1.6 nm ($p<0.0001$) on exposure of the sacs to 1 mM oleate. Immunogold-EM using anti-Caveolin-1 antibodies showed multiple beads over only vesicles in both the fasted and 1 mM oleate fed cytosol. No beads were seen if IgG was

used. Conclusions: Absorbed dietary FA are present in rat intestinal cytosol in Caveolin-1 containing lipid rafts not in FABP as has been proposed previously. The FA are thus sequestered from interacting with enterocyte membranes. We speculate that the FA enter the caveolae at the apical membrane, the caveolae detach from the apical membrane to enter the cytosol and are then targeted to the endoplasmic reticulum (ER) for incorporation into complex lipids.

3.1662 Effector Granules in Human T Lymphocytes: Proteomic Evidence for Two Distinct Species of Cytotoxic Effector Vesicles

Schmidt, H., Gelhaus, C., Nebendahl, M., Lettau, M., Lucius, R., Leippe, M., Kabelitz, D. and janssen, O.
J. Proteome Res., ***10(4)***, *1603-1620 (2011)*

Cytotoxic T cells mobilize effector proteins from prestored lysosomal compartments. Since different activation signals result in alternative routes of target cell killing, utilizing either FasL or the granzyme B/perforin pathway, the existence of distinct forms of effector granules was recently suggested. Applying a protocol for the separation of intact organelles from activated T lymphoblasts, we noticed that FasL-associated secretory lysosomes (SL) segregate from vesicles containing larger amounts of granzymes and granulysin. We previously analyzed the proteome of secretory lysosomes from NK and T cells and now describe the proteome of granzyme-containing vesicles. Moreover, intact FasL-associated SL and granzyme-containing vesicles were compared by electron microscopy and respective extracts were characterized by Western blotting. With the present report, we provide a comprehensive proteome map of granzyme-containing granules and unequivocally demonstrate that T lymphoblasts contain at least two distinct types of effector vesicles. Moreover, the overall protein content of the two vesicle populations was compared by 2D difference gel electrophoresis. Interestingly, the observed differences in protein distribution were not restricted to effector proteins but also applied to cytoskeleton-associated elements that could argue for a differential transport or initiation of degranulation. To our knowledge, this is the first comprehensive description of distinct effector granules in T cells.

3.1663 Identification, characterization and regulation studies of the aconitase of *Paracoccidioides brasiliensis*

De A. Brito, W., Rezende, T.C.V., Parente, A.F., Ricart, C.A.O., de Sousa, M.V., Bao, S.N. and de A. Soares, C.M.
Fungal Biol., ***115***, *697-707 (2011)*

A protein species preferentially expressed in yeast cells with a molecular mass of 80 kDa and isoeletric point (p*I*) of 7.79 was isolated from the proteome of *Paracoccidioides brasiliensis* and characterized as an aconitase (ACO) (E.C. 4.2.1.3). ACO is an enzyme that catalyzes the isomerization of citrate to isocitrate in both the Krebs cycle and the glyoxylate cycle. We report the cloning and characterization of the cDNA encoding the ACO of *P. brasiliensis* (*Pb*ACO). The cDNA showed a 2361 bp open reading frame (ORF) and encoded a predicted protein with 787 amino acids. Polyclonal antibodies against the purified recombinant *Pb*ACO was obtained in order to analyze the subcellular localization of the molecule in *P. brasiliensis*. The protein is present in the extracellular fluid, cell wall enriched fraction, mitochondria, cytosol and peroxisomes of yeast cells as demonstrated by western blot and immunocytochemistry analysis. The expression analysis of the *Pbaco* gene was performed by quantitative real-time RT-PCR and results demonstrated an increased expression in yeast cells compared to mycelia. Real-time RT-PCR assays was also used to evaluate the *Pbaco* expression when the fungus grows on media with acetate and ethanol as sole carbon sources and in different iron levels. The results demonstrated that *Pbaco* transcript is over expressed in acetate and ethanol as sole carbon sources and in high-iron conditions.

3.1664 Challenges and solutions for the identification of membrane proteins in non-model plants

Vertommen, A., Panis, B., Swennen, R. and Carpentier, S.C.
J. Proteomics, ***74***, *1165-1181 (2011)*

The workhorse for proteomics in non-model plants is classical two-dimensional electrophoresis, a combination of iso-electric focusing and SDS-PAGE. However, membrane proteins with multiple membrane spanning domains are hardly detected on classical 2-DE gels because of their low abundance and poor solubility in aqueous media. In the current review, solutions that have been proposed to handle these two problems in non-model plants are discussed. An overview of alternative techniques developed for membrane proteomics is provided together with a comparison of their strong and weak points. Subsequently, strengths and weaknesses of the different techniques and methods to evaluate the

identification of membrane proteins are discussed. Finally, an overview of recent plant membrane proteome studies is provided with the used separation technique and the number of identified membrane proteins listed.

3.1665 Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles
György, B., Szabo, T.G., Pasztoi, M., Pal, Z., Misjak, P., Aradi, B., Laslo, V., Pallinger, E., Pap, E., Kittel, A., Nagy, G., Falus, A. and Buzas, E.I.
Celll. Mol. Life Sci., ***68(16)***, *2667-2688 (2011)*

Abstract Release of membrane vesicles, a process conserved in both prokaryotes and eukaryotes, represents an evolutionary link, and suggests essential functions of adynamic extracellular vesicular compartment (including exosomes, microparticles or microvesicles and apoptotic bodies). Compelling evidence supports the significance of this compartment in a broad range of physiological and pathological processes. However, classification of membrane vesicles, protocols of their isolation and detection, molecular details of vesicular release, clearance and biological functions are still under intense investigation. Here, we give a comprehensive overview of extracellular vesicles. After discussing the technical pitfalls and potential
artifacts of the rapidly emerging field, we compare results from meta-analyses of published proteomic studies on membrane vesicles. We also summarize clinical implications of membrane vesicles. Lessons from this compartment challenge current paradigms concerning the mechanisms of intercellular communication and immune regulation. Furthermore, its clinical implementation may open new perspectives in translational medicine both in diagnostics and therapy.

3.1666 A Concerted Action of Hepatitis C Virus P7 and Nonstructural Protein 2 Regulates Core Localization at the Endoplasmic Reticulum and Virus Assembly
Boson, B., Granio, O., Bartenschlager, R. and Cosset, F-L.
PloS Pathogens, ***7(7)***, *e1002144 (2011)*

Hepatitis C virus (HCV) assembly remains a poorly understood process. Lipid droplets (LDs) are thought to act as platforms for the assembly of viral components. The JFH1 HCV strain replicates and assembles in association with LD-associated membranes, around which viral core protein is predominantly detected. In contrast, despite its intrinsic capacity to localize to LDs when expressed individually, we found that the core protein of the high-titer Jc1 recombinant virus was hardly detected on LDs of cell culture-grown HCV (HCVcc)-infected cells, but was mainly localized at endoplasmic reticulum (ER) membranes where it colocalized with the HCV envelope glycoproteins. Furthermore, high-titer cell culture-adapted JFH1 virus, obtained after long-term culture in Huh7.5 cells, exhibited an ER-localized core in contrast to non-adapted JFH1 virus, strengthening the hypothesis that ER localization of core is required for efficient HCV assembly. Our results further indicate that p7 and NS2 are HCV strain-specific factors that govern the recruitment of core protein from LDs to ER assembly sites. Indeed, using expression constructs and HCVcc recombinant genomes, we found that p7 is sufficient to induce core localization at the ER, independently of its ion-channel activity. Importantly, the combined expression of JFH1 or Jc1 p7 and NS2 induced the same differential core subcellular localization detected in JFH1- *vs.* Jc1-infected cells. Finally, results obtained by expressing p7-NS2 chimeras between either virus type indicated that compatibilities between the p7 and the first NS2 trans-membrane domains is required to induce core-ER localization and assembly of extra- and intra-cellular infectious viral particles. In conclusion, we identified p7 and NS2 as key determinants governing the subcellular localization of HCV core to LDs *vs.* ER and required for initiation of the early steps of virus assembly.

3.1667 Isolation of detergent-resistant membranes from plant photosynthetic and non-photosynthetic tissues
Carmona-Salazar, L., El Hafidi, M., Enriquez-Arredondo, C., Vazquez-Vazquez, C., Gonzales de la Vera, C. and Gavilanes-Ruiz, M.
Anal. Biochem., ***417***, *220-227 (2011)*

Microdomains, or lipid rafts, are transient membrane regions enriched in sphingolipids and sterols that have only recently, but intensively, been studied in plants. In this work, we report a detailed, easy-to-follow, and fast procedure to isolate detergent-resistant membranes (DRMs) from purified plasma membranes (PMs) that was used to obtain DRMs from *Phaseolus vulgaris* and *Nicotiana tabacum* leaves and germinating *Zea mays* embryos. Characterized according to yield, ultrastructure, and sterol composition, these DRM preparations showed similarities to analogous preparations from other eukaryotic

cells. Isolation of DRMs from germinating maize embryos reveals the presence of microdomains at very early developmental stages of plants.

3.1668 An N-terminal Polybasic Domain and Cell Surface Localization Are Required for Mutant Prion Protein Toxicity

Solomon, I.H., Khatri, N., Biasini, E., Massignan, T., Huettner, J.E. and Harris, D.A.
J. Biol. Chem., ***286(16)***, *14724-14736 (2011)*

There is evidence that alterations in the normal physiological activity of PrP^{C} contribute to prion-induced neurotoxicity. This mechanism has been difficult to investigate, however, because the normal function of PrP^{C} has remained obscure, and there are no assays available to measure it. We recently reported that cells expressing PrP deleted for residues 105–125 exhibit spontaneous ionic currents and hypersensitivity to certain classes of cationic drugs. Here, we utilize cell culture assays based on these two phenomena to test how changes in PrP sequence and/or cellular localization affect the functional activity of the protein. We report that the toxic activity of Δ105–125 PrP requires localization to the plasma membrane and depends on the presence of a polybasic amino acid segment at the N terminus of PrP. Several different deletions spanning the central region as well as three disease-associated point mutations also confer toxic activity on PrP. The sequence domains identified in our study are also critical for PrP^{Sc} formation, suggesting that common structural features may govern both the functional activity of PrP^{C} and its conversion to PrP^{Sc}.

3.1669 Ubp15p, a Ubiquitin Hydrolase Associated with the Peroxisomal Export Machinery

Debelyy, M.O., Platta, H.W., Saffian, D., hensel, A., thoms, S., Meyer, H.E., Warschied, B., Gizalsky, W. and Erdmann, R.
J. Biol. Chem., ***286(32)***, *28223-28234 (2011)*

Peroxisomal matrix protein import is facilitated by cycling receptors shuttling between the cytosol and the peroxisomal membrane. One crucial step in this cycle is the ATP-dependent release of the receptors from the peroxisomal membrane. This step is facilitated by the peroxisomal AAA (ATPases associated with various cellular activities) proteins Pex1p and Pex6p with ubiquitination of the receptor being the main signal for its export. Here we report that the AAA complex contains dislocase as well as deubiquitinating activity. Ubp15p, a ubiquitin hydrolase, was identified as a novel constituent of the complex. Ubp15p partially localizes to peroxisomes and is capable of cleaving off ubiquitin moieties from the type I peroxisomal targeting sequence (PTS1) receptor Pex5p. Furthermore, Ubp15p-deficient cells are characterized by a stress-related PTS1 import defect. The results merge into a picture in which removal of ubiquitin from the PTS1 receptor Pex5p is a specific event and might represent a vital step in receptor recycling.

3.1670 Urban planning of the endoplasmic reticulum (ER): How diverse mechanisms segregate the many functions of the ER

Lynes, E.M. and Simmen, T.
Biochim. Biophys. Acta, ***1813***, *1893-1905 (2011)*

The endoplasmic reticulum (ER) is the biggest organelle in most cell types, but its characterization as an organelle with a continuous membrane belies the fact that the ER is actually an assembly of several, distinct membrane domains that execute diverse functions. Almost 20 years ago, an essay by Sitia and Meldolesi first listed what was known at the time about domain formation within the ER. In the time that has passed since, additional ER domains have been discovered and characterized. These include the mitochondria-associated membrane (MAM), the ER quality control compartment (ERQC), where ER-associated degradation (ERAD) occurs, and the plasma membrane-associated membrane (PAM). Insight has been gained into the separation of nuclear envelope proteins from the remainder of the ER. Research has also shown that the biogenesis of peroxisomes and lipid droplets occurs on specialized membranes of the ER. Several studies have shown the existence of specific marker proteins found on all these domains and how they are targeted there. Moreover, a first set of cytosolic ER-associated sorting proteins, including phosphofurin acidic cluster sorting protein 2 (PACS-2) and Rab32 have been identified. Intra-ER targeting mechanisms appear to be superimposed onto ER retention mechanisms and rely on transmembrane and cytosolic sequences. The crucial roles of ER domain formation for cell physiology are highlighted with the specific targeting of the tumor metastasis regulator gp78 to ERAD-mediating membranes or of the promyelocytic leukemia protein to the MAM.

3.1671 A stable yeast strain efficiently producing cholesterol instead of ergosterol is functional for tryptophan uptake, but not weak organic acid resistance
Souza, C.M., Schwabe, T.M.E., Pichler, H., Ploier, B., Leitner, E., Guan, X.L., Wenk, M.R., Riezman, I. and Riezman, H.
*Metabolic Engineering, **13**, 555-569 (2011)*

Sterols are major lipids in eukaryotes and differ in their specific structure between species. Both cholesterol and ergosterol can form liquid ordered domains in artificial membranes. We reasoned that substituting the main sterol ergosterol by cholesterol in yeast should permit domain formation and discriminate between physical and sterol structure-dependent functions. Using a cholesterol-producing yeast strain, we show that solute transporters for tryptophan and arginine are functional, whereas the export of weak organic acids via Pdr12p, a multi-drug resistance family member, is not. The latter reveals a sterol function that is probably dependent upon a precise sterol structure. We present a series of novel yeast strains with different sterol compositions as valuable tools to characterize sterol function and use them to refine the sterol requirements for Pdr12p. These strains will also be improved hosts for heterologous expression of sterol-dependent proteins and safe sources to obtain pure cholesterol and other sterols.

3.1672 Fatty Acid and Peptide Profiles in Plasma Membrane and Membrane Rafts of PUFA Supplemented RAW264.7 Macrophages
Schumann, J., Leichtle, A., Thiery, J. and Fuhrmann, H.
*PloS One, **6(8)**, e24066 (2011)*

The eukaryotic cell membrane possesses numerous complex functions, which are essential for life. At this, the composition and the structure of the lipid bilayer are of particular importance. Polyunsaturated fatty acids may modulate the physical properties of biological membranes via alteration of membrane lipid composition affecting numerous physiological processes, e.g. in the immune system. In this systematic study we present fatty acid and peptide profiles of cell membrane and membrane rafts of murine macrophages that have been supplemented with saturated fatty acids as well as PUFAs from the n-3, the n-6 and the n-9 family. Using fatty acid composition analysis and mass spectrometry-based peptidome profiling we found that PUFAs from both the n-3 and the n-6 family have an impact on lipid and protein composition of plasma membrane and membrane rafts in a similar manner. In addition, we found a relation between the number of bis-allyl-methylene positions of the PUFA added and the unsaturation index of plasma membrane as well as membrane rafts of supplemented cells. With regard to the proposed significance of lipid microdomains for disease development and treatment our study will help to achieve a targeted dietary modulation of immune cell lipid bilayers.

3.1673 Specific binding of activated Vip3Aa10 to Helicoverpa armigera brush border membrane vesicles results in pore formation
Liu, J-G., Yang, A-Z., Shen, X-H., Hua, B-G. and Shi, G-L.
*J. Invertebrate Pathol., **108**, 92-97 (2011)*

Helicoverpa armigera is one of the most harmful pests in China. Although it had been successfully controlled by Cry1A toxins, some *H. armigera* populations are building up resistance to Cry1A toxins in the laboratory. Vip3A, secreted by *Bacillus thuringiensis*, is another potential toxin against *H. armigera*. Previous reports showed that activated Vip3A performs its function by inserting into the midgut brush border membrane vesicles (BBMV) of susceptible insects. To further investigate the binding of Vip3A to BBMV of *H. armigera*, the full-length Vip3Aa10 toxin expressed in *Escherichia coli* was digested by trypsin or midgut juice extract, respectively. Among the fragments of digested Vip3Aa10, only a 62 kDa fragment (Vip3Aa10-T) exhibited binding to BBMV of *H. armigera* and has insecticidal activity. Moreover, this interaction was specific and was not affected by the presence of Cry1Ab toxin. Binding of Vip3Aa10-T to BBMV resulted in the formation of an ion channel. Unlike Cry1A toxins, Vip3Aa10-T was just slightly associated with lipid rafts of BBMV. These data suggest that although activated Vip3Aa10 specifically interacts with BBMV of *H. armigera* and forms an ion channel, the mode of action of it may be different from that of Cry1A toxins.

3.1674 Biochemical Characterization of APPL Endosomes: The Role of Annexin A2 in APPL Membrane Recruitment
Urbanska, A., Sadowski, L., Kalaidzidis, Y. and Miaczynska, M.
*Traffic, **12(9)**, 1227-1241 (2011)*

APPL endosomes are a recently identified subpopulation of early endosomes characterized by the presence of two homologous Rab5 effector proteins APPL1 and APPL2. They exhibit only limited colocalization with EEA1, another Rab5 effector and a marker of the canonical early endosomes. Although APPL endosomes appear to play important roles in cargo trafficking and signal transduction, their protein composition and biochemical properties remain largely unknown. Here we employed membrane fractionation methods to characterize APPL endosomes biochemically. We demonstrate that they represent heterogeneous membrane structures which can be discriminated from the canonical EEA1-positive early endosomes by their partly different physical properties and a distinct migration pattern in the continuous density gradients. In search for other potential markers of APPL endosomes we identified Annexin A2 as an interacting partner of both APPL1 and APPL2. Annexin A2 is a Ca^{2+} and phosphatidylinositol 4,5-bisphosphate binding protein, previously implicated in several endocytic steps. We show that Annexin A2 co-fractionates and colocalizes with APPL endosomes. Moreover, silencing of its expression causes solubilization of APPL2 from endosomes. Although Annexin A2 is not an exclusive marker of APPL endosomes, our data suggest that it has an important function in membrane recruitment of APPL proteins, acting in parallel to Rab5.

3.1675 Curcumin Inhibition of the Functional Interaction between Integrin α6β4 and the Epidermal Growth Factor Receptor

Soung, Y.H: and Chung, J.

Mol. Cancer Ther., ***10***, *883-891 (2011)*

The functional interaction between integrin α6β4 and growth factor receptors has been implicated in key signaling pathways important for cancer cell function. However, few attempts have been made to selectively target this interaction for therapeutic intervention. Previous studies showed that curcumin, a yellow pigment isolated from turmeric, inhibits integrin α6β4 signaling important for breast carcinoma cell motility and invasion, but the mechanism is not currently known. To address this issue, we tested the hypothesis that curcumin inhibits the functional interaction between α6β4 and the epidermal growth factor receptor (EGFR). In this study, we found that curcumin disrupts functional and physical interactions between α6β4 and EGFR, and blocks α6β4/EGFR-dependent functions of carcinoma cells expressing the signaling competent form of α6β4. We further showed that curcumin inhibits EGF-dependent mobilization of α6β4 from hemidesmosomes to the leading edges of migrating cells such as lammelipodia and filopodia, and thereby prevents α6β4 distribution to lipid rafts where functional interactions between α6β4 and EGFR occur. These data suggest a novel paradigm in which curcumin inhibits α6β4 signaling and functions by altering intracellular localization of α6β4, thus preventing its association with signaling receptors such as EGFR.

3.1676 Plant organelle proteomics: Collaborating for optimal cell function

Agrawal, G.K: et al

Mass Spectrometry Reviews, ***30(5)****, 772-853 (2011)*

Organelle proteomics describes the study of proteins present in organelle at a particular instance during the whole period of their life cycle in a cell. Organelles are specialized membrane bound structures within a cell that function by interacting with cytosolic and luminal soluble proteins making the protein composition of each organelle dynamic. Depending on organism, the total number of organelles within a cell varies, indicating their evolution with respect to protein number and function. For example, one of the striking differences between plant and animal cells is the plastids in plants. Organelles have their own proteins, and few organelles like mitochondria and chloroplast have their own genome to synthesize proteins for specific function and also require nuclear-encoded proteins. Enormous work has been performed on animal organelle proteomics. However, plant organelle proteomics has seen limited work mainly due to: (i) inter-plant and inter-tissue complexity, (ii) difficulties in isolation of subcellular compartments, and (iii) their enrichment and purity. Despite these concerns, the field of organelle proteomics is growing in plants, such as Arabidopsis, rice and maize. The available data are beginning to help better understand organelles and their distinct and/or overlapping functions in different plant tissues, organs or cell types, and more importantly, how protein components of organelles behave during development and with surrounding environments. Studies on organelles have provided a few good reviews, but none of them are comprehensive. Here, we present a comprehensive review on plant organelle proteomics starting from the significance of organelle in cells, to organelle isolation, to protein identification and to biology and beyond. To put together such a systematic, in-depth review and to translate acquired knowledge in a proper and adequate form, we join minds to provide discussion and viewpoints on the collaborative nature of organelles in cell, their proper function and evolution.

3.1677 Function of MRP1/ABCC1 is not dependent on cholesterol or cholesterol-stabilized lipid rafts
Meszaros, P., Klappe, K., Hummel, I., Hoekstra, D. and Kok, J.W.
Biochem. J., ***437***, *483-491 (2011)*

MRP1 (multidrug-resistance-related protein 1)/ABCC1 (ATP-binding cassette transporter C1) has been localized in cholesterol-enriched lipid rafts, which suggests a role for these lipid rafts and/or cholesterol in MRP1 function. In the present study, we have shown for the first time that nearly complete oxidation of free cholesterol in the plasma membrane of BHK-MRP1 (MRP1-expressing baby hamster kidney) cells did not affect MRP1 localization in lipid rafts or its efflux function, using 5-carboxyfluorescein diacetate as a substrate. Inhibition of cholesterol biosynthesis, using lovastatin in combination with RO 48-8071, an inhibitor of oxidosqualene cyclase, resulted in a shift of MRP1 out of lipid raft fractions, but did not affect MRP1-mediated efflux in Neuro-2a (neuroblastoma) cells. Short-term methyl-β-cyclodextrin treatment was equally effective in removing free cholesterol from Neuro-2a and BHK-MRP1 cells, but affected MRP1 function only in the latter. The kinetics of loss of both MRP1 efflux function and lipid raft association during long-term methyl-β-cyclodextrin treatment did not match the kinetics of free cholesterol removal in both cell lines. Moreover, MRP1 activity was measured in vesicles consisting of membranes isolated from BHK-MRP1 cells using the substrate cysteinyl leukotriene C_4 and was not changed when the free cholesterol level of these membranes was either decreased or increased. In conclusion, MRP1 activity is not correlated with the level of free cholesterol or with localization in cholesterol-dependent lipid rafts.

3.1678 Differential, Type I Interferon-Mediated Autophagic Trafficking of Hepatitis C Virus Proteins in Mouse Liver
Desai, M.M., Gong, B., Chan, T., Davey, R.A., Soong, L., Kolokoltsov, A.A. and Sun, J.
Gastroenterology, ***141(2)****, 674-685 (2011)*

Background & Aims
The hepatitis C virus (HCV) serine protease NS3/4A can cleave mitochondria-associated antiviral signaling protein (MAVS) and block retinoic acid–inducible gene I–mediated interferon (IFN) responses. Although this mechanism is thought to have an important role in HCV-mediated innate immunosuppression, its significance in viral persistence is not clear.
Methods
We generated transgenic mice that express the HCV NS3/4A proteins specifically in the liver and challenged the animals with a recombinant vesicular stomatitis virus, a synthetic HCV genome, IFN alfa, or IFN beta. We evaluated the effects of HCV serine protease on the innate immune responses and their interactions.
Results
Expression of HCV NS3/4A resulted in cleavage of intrahepatic MAVS; challenge of transgenic mice with vesicular stomatitis virus or a synthetic HCV genome induced strong, type I IFN-mediated responses that were not significantly lower than those of control mice. Different challenge agents induced production of different ratios of IFN alfa and beta, resulting in different autophagic responses and vesicular trafficking patterns of endoplasmic reticulum- and mitochondria-associated viral proteins. IFN beta promoted degradation of the viral proteins by the autolysosome. Variant isoforms of MAVS were associated with distinct, type I IFN-mediated autophagic responses; these responses have a role in trafficking of viral components to endosomal compartments that contain Toll-like receptor–3.
Conclusions
IFN beta mediates a distinct autophagic mechanism of antiviral host defense. MAVS has an important role in type I IFN-induced autophagic trafficking of viral proteins.

3.1679 Positive Feedback Loop Between PI3K-Akt-mTORC1 Signaling and the Lipogenic Pathway Boosts Akt Signaling: Induction of the Lipogenic Pathway by a Melanoma Antigen
Yamauchi, Y., Furukawa, K, Hamamura, K. et al
Cancer Res., ***71****, 4989-4997 (2011)*

The lipogenic phenotype is a metabolic hallmark of cancer cells. Sterol regulatory element–binding proteins (SREBP) are key transcriptional factors to regulate biosynthesis of cholesterol and fatty acids. It has been poorly understood how the lipogenic phenotype in cancer cells is regulated and how it augments their malignant properties. Here we describe roles of the melanoma antigen ganglioside GD3 and phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR complex 1 (mTORC1) signaling in the regulation of SREBP activity, cholesterol biosynthesis, and the integrity of lipid rafts in human melanoma cells. GD3

expression induced the activation of both SREBP-1 and SREBP-2. Consequently, HMG-CoA reductase expression and cholesterol biosynthesis increased. The activation of the SREBP pathway was independent of the oncogenic BRAF mutation. On the other hand, it was regulated by PI3K-Akt-mTORC1 signaling in human melanoma cells. Disruption of the signaling pathway resulted in the reduction of cholesterol in lipid rafts. Inhibition of the SREBP pathway attenuated Akt activation in lipid rafts and suppressed the growth of human melanoma cells *in vitro* and *in vivo*. These results suggest that PI3K-Akt-mTORC1 signaling is important for the integrity of lipid rafts by regulating SREBP activation and subsequent cholesterogenesis. We thus propose a positive feedback circuit in which PI3K-Akt-mTORC1-SREBP signaling boosts Akt signaling in human melanoma cells expressing GD3.

3.1680 HIV-1 gp41 ectodomain enhances *Cryptococcus neoformans* binding to human brain microvascular endothelial cells via gp41 core-induced membrane activities
Huang, S-H., Wu, C-H., Jiang, S., Bahner, I., Lossinsky, A.S. and Jong, A.Y.
Biochem. J., ***438***, *457-466 (2011)*

Cryptococcus neoformans causes life-threatening meningoencephalitis, particularly prevalent in AIDS patients. The interrelationship between *C. neoformans* and HIV-1 is intriguing, as both pathogens elicit severe neuropathological complications. We have previously demonstrated that the HIV-1 gp41 ectodomain fragments gp41-I33 (amino acids 579–611) and gp41-I90 (amino acids 550–639) can enhance *C. neoformans* binding to HBMECs (human brain microvascular endothelial cells). Both peptides contain the loop region of gp41. In the present study, we used immunofluorescence microscopy and transmission and scanning electron microscopy to explore the underlying mechanisms. Our findings indicated that both *C. neoformans* and gp41-I90 up-regulated ICAM-1 (intercellular adhesion molecule 1) on the HBMECs and elicited membrane ruffling on the surface of HBMECs. The HIV-1 gp41 ectodomain could also induce CD44 and β-actin redistribution to the membrane lipid rafts, but it could not enhance PKCα (protein kinase Cα) phosphorylation like *C. neoformans*. Instead, gp41-I90 was able to induce syncytium formation on HBMECs. The results of the present study suggest HIV-1 gp41-enhanced *C. neoformans* binding to HBMECs via gp41 core domain-induced membrane activities, revealing a potential mechanism of invasion for this pathogenic fungus into the brain tissues of HIV-1-infected patients.

3.1681 Protein Transfer Into Human Cells by VSV-G-induced Nanovesicles
Mangeot, P-E., Dollet, S., Girard, M., Ciancia, C., Joly, S., Peschanski, M. and Lotteau, V.
Molecular Therapy, ***19(9)***, *1656-1666 (2011)*

Identification of new techniques to express proteins into mammal cells is of particular interest for both research and medical purposes. The present study describes the use of engineered vesicles to deliver exogenous proteins into human cells. We show that overexpression of the spike glycoprotein of the vesicular stomatitis virus (VSV-G) in human cells induces the release of fusogenic vesicles named gesicles. Biochemical and functional studies revealed that gesicles incorporated proteins from producer cells and could deliver them to recipient cells. This protein-transduction method allows the direct transport of cytoplasmic, nuclear or surface proteins in target cells. This was demonstrated by showing that the TetR transactivator and the receptor for the murine leukemia virus (MLV) envelope [murine cationic amino acid transporter-1 (mCAT-1)] were efficiently delivered by gesicles in various cell types. We further shows that gesicle-mediated transfer of mCAT-1 confers to human fibroblasts a robust permissiveness to ecotropic vectors, allowing the generation of human-induced pluripotent stem cells in level 2 biosafety facilities. This highlights the great potential of mCAT-1 gesicles to increase the safety of experiments using retro/lentivectors. Besides this, gesicles is a versatile tool highly valuable for the nongenetic delivery of functions such as transcription factors or genome engineering agents.

3.1682 Inhibition of Mucin O-Glycosylation Promotes Endocytosis and Nanoparticle Uptake in Corneal Epithelial Cells
Argeso, P., Guzman-Aranguez, A., Woodward, A. and Pintor, J.J.
Invest. Ophthalmol. Vis. Sci., ***52***, *E-abstract 4394 (2011)*

Purpose: Recent evidence has shown that O-glycans on cell surface-associated mucins contribute to maintaining barrier function by interacting with β-galactoside-binding lectins on the epithelial glycocalyx; however, the mechanisms involved have not been completely characterized. In this work, we have evaluated whether abrogation of O-glycosylation promotes endocytosis and particle uptake in human corneal epithelial (HCLE) cells.

Methods: Downregulation of mucin O-glycosylation in HCLE cells was carried out using a stable tetracycline-inducible RNA interfering system to knockdown c1galt1, a critical galactosyltransferase required for the synthesis of core 1 O-glycans. Subcellular membrane vesicles were fractionated by ultracentrifugation in a 5-20% (w/v) continuous gradient of **iodixanol**. Fractions were analyzed by western blot using a mouse monoclonal MUC16 antibody. HCLE cells were incubated with 0.1 μm carboxylate-modified fluorescent nanospheres, and uptake analyzed by confocal microscopy and fluorometry before and after inhibition of endocytosis. Tight junction integrity was evaluated using transepithelial electrical resistance and ZO-1 staining.
Results: Fractionation of membrane fragments revealed that trafficking of the cell surface mucin MUC16 was altered in cells transfected with c1galt1 shRNA. Moreover, in particle internalization studies, c1galt1 shRNA-transfected cells had a 1.63-fold increase in nanoparticle uptake as compared to scramble control. Nanoparticle internalization was dramatically reduced at 4°C, when active transport processes are blocked, and by sodium azide, a general inhibitor of endocytic processes. Dynasore and nocodazole significantly reduced nanoparticle uptake by 37% and 36%, respectively, suggesting a mechanism for coated pit budding and vesicular trafficking in nanoparticle uptake. The involvement of the clathrin-mediated pathway was supported by a significant decrease in uptake observed with chlorpromazine (44%) and hypertonic media (53%). Downregulation of c1galt1 expression did not decrease the transepithelial electrical resistance or ZO-1 staining, indicating that increased nanoparticle uptake occurs through the transcellular pathway.
Conclusions: These results indicate that mucin O-glycans hinder endocytosis and nanoparticle uptake, and suggest that transient manipulation of the glycocalyx barrier is an alternative approach to delivering therapeutic nanoparticles to the cornea.

3.1683 Cardiac ATP-sensitive K+ channel associates with the glycolytic enzyme complex
Hong, M., Kefaloyianni, E., Bao, L., Malester, B., Delaroche, D., Neubert, T.A. and Coetzee, W.A.
FASEB J. ***25(7)****, 2456-2467 (2011)*

Being gated by high-energy nucleotides, cardiac ATP-sensitive potassium (K_{ATP}) channels are exquisitely sensitive to changes in cellular energy metabolism. An emerging view is that proteins associated with the K_{ATP} channel provide an additional layer of regulation. Using putative sulfonylurea receptor (SUR) coiled-coil domains as baits in a 2-hybrid screen against a rat cardiac cDNA library, we identified glycolytic enzymes (GAPDH and aldolase A) as putative interacting proteins. Interaction between aldolase and SUR was confirmed using GST pulldown assays and coimmunoprecipitation assays. Mass spectrometry of proteins from K_{ATP} channel immunoprecipitates of rat cardiac membranes identified glycolysis as the most enriched biological process. Coimmunoprecipitation assays confirmed interaction for several glycolytic enzymes throughout the glycolytic pathway. Immunocytochemistry colocalized many of these enzymes with K_{ATP} channel subunits in rat cardiac myocytes. The catalytic activities of aldolase and pyruvate kinase functionally modulate K_{ATP} channels in patch-clamp experiments, whereas d-glucose was without effect. Overall, our data demonstrate close physical association and functional interaction of the glycolytic process (particularly the distal ATP-generating steps) with cardiac K_{ATP} channels.

3.1684 The Assembly of Proline-rich Membrane Anchor (PRiMA)-linked Acetylcholinesterase Enzyme: GLYCOSYLATION IS REQUIRED FOR ENZYMATIC ACTIVITY BUT NOT FOR OLIGOMERIZATION
Chen, V.P., Choi, R.C.Y., Chan, W.K., Leung, K.W., Guo, A.J.Y., Chan, G.K.L., Luk, W.K.W. and Tsim, K.W.K.
J. Biol. Chem., ***286(38)****, 32948-32961 (2011)*

Acetylcholinesterase (AChE) anchors onto cell membranes by a transmembrane protein PRiMA (proline-rich membrane anchor) as a tetrameric form in vertebrate brain. The assembly of AChE tetramer with PRiMA requires the C-terminal "t-peptide" in AChE catalytic subunit ($AChE_T$). Although mature AChE is well known *N*-glycosylated, the role of glycosylation in forming the physiologically active PRiMA-linked AChE tetramer has not been studied. Here, several lines of evidence indicate that the *N*-linked glycosylation of $AChE_T$ plays a major role for acquisition of AChE full enzymatic activity but does not affect its oligomerization. The expression of the $AChE_T$ mutant, in which all *N*-glycosylation sites were deleted, together with PRiMA in HEK293T cells produced a glycan-depleted PRiMA-linked AChE tetramer but with a much higher K_m value as compared with the wild type. This glycan-depleted enzyme was assembled in endoplasmic reticulum but was not transported to Golgi apparatus or plasma membrane.

3.1685 Molecular Determinants of Ciliary Membrane Localization of Trypanosoma cruzi Flagellar Calcium-binding Protein

Maric, D., McGwire, B.S., Buchanan, K.T., Olson, C.L., Emmer, B.T., Epting, C.L. and Engman, D.M.
J. Biol. Chem., ***286(38)***, *33109-33117 (2011)*

The flagellar calcium-binding protein (FCaBP) of *Trypanosoma cruzi* is localized to the flagellar membrane in all life cycle stages of the parasite. Myristoylation and palmitoylation of the N terminus of FCaBP are necessary for flagellar membrane targeting. Not all dually acylated proteins in *T. cruzi* are flagellar, however. Other determinants of FCaBP therefore likely contribute to flagellar specificity. We generated *T. cruzi* transfectants expressing the N-terminal 24 or 12 amino acids of FCaBP fused to GFP. Analysis of these mutants revealed that although amino acids 1–12 are sufficient for dual acylation and membrane binding, amino acids 13–24 are required for flagellar specificity and lipid raft association. Mutagenesis of several conserved lysine residues in the latter peptide demonstrated that these residues are essential for flagellar targeting and lipid raft association. Finally, FCaBP was expressed in the protozoan *Leishmania amazonensis*, which lacks FCaBP. The flagellar localization and membrane association of FCaBP in *L. amazonensis* suggest that the mechanisms for flagellar targeting, including a specific palmitoyl acyltransferase, are conserved in this organism.

3.1686 Triage of oxidation-prone proteins by Sqstm1/p62 within the mitochondria

Lee, M. and Shin, J.
Biochem. Biophys. Acta, ***413***, *122-127 (2011)*

As the mitochondrion is vulnerable to oxidative stress, cells have evolved several strategies to maintain mitochondrial integrity, including mitochondrial protein quality control mechanisms and autophagic removal of damaged mitochondria. Involvement of an autophagy adaptor, Sqstm1/p62, in the latter process has been recently described. In the present study, we provide evidence that a portion of p62 directly localizes within the mitochondria and supports stable electron transport by forming heterogeneous protein complexes. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) of mitochondrial proteins co-purified with p62 revealed that p62 interacts with several oxidation-prone proteins, including a few components of the electron transport chain complexes, as well as multiple chaperone molecules and redox regulatory enzymes. Accordingly, p62-deficient mitochondria exhibited compromised electron transport, and the compromised function was partially restored by *in vitro* delivery of p62. These results suggest that p62 plays an additional role in maintaining mitochondrial integrity at the vicinity of target machineries through its function in relation to protein quality control.

3.1687 Mammalian ACSF3 Protein Is a Malonyl-CoA Synthetase That Supplies the Chain Extender Units for Mitochondrial Fatty Acid Synthesis

Witkowski, A., Thweatt, J. and Smith, S.
J. Biol. Chem., ***286(39)***, *33729-33736 (2011)*

The objective of this study was to identify a source of intramitochondrial malonyl-CoA that could be used for *de novo* fatty acid synthesis in mammalian mitochondria. Because mammalian mitochondria lack an acetyl-CoA carboxylase capable of generating malonyl-CoA inside mitochondria, the possibility that malonate could act as a precursor was investigated. Although malonyl-CoA synthetases have not been identified previously in animals, interrogation of animal protein sequence databases identified candidates that exhibited sequence similarity to known prokaryotic forms. The human candidate protein ACSF3, which has a predicted N-terminal mitochondrial targeting sequence, was cloned, expressed, and characterized as a 65-kDa acyl-CoA synthetase with extremely high specificity for malonate and methylmalonate. An arginine residue implicated in malonate binding by prokaryotic malonyl-CoA synthetases was found to be positionally conserved in animal ACSF3 enzymes and essential for activity. Subcellular fractionation experiments with HEK293T cells confirmed that human ACSF3 is located exclusively in mitochondria, and RNA interference experiments verified that this enzyme is responsible for most, if not all, of the malonyl-CoA synthetase activity in the mitochondria of these cells. In conclusion, unlike fungi, which have an intramitochondrial acetyl-CoA carboxylase, animals require an alternative source of mitochondrial malonyl-CoA; the mitochondrial ACSF3 enzyme is capable of filling this role by utilizing free malonic acid as substrate.

3.1688 N-Glycans and Glycosylphosphatidylinositol-Anchor Act on Polarized Sorting of Mouse PrPC in Madin-Darby Canine Kidney Cells

Puig, B., Altmeppen, H.C., Thurm, D., Geissen, M., Conrad, C., Braulke, T. and Glatsel, M.

*PloS One, **6(9)**, e24624 (2011)*

The cellular prion protein (PrP^C) plays a fundamental role in prion disease. PrP^C is a glycosylphosphatidylinositol (GPI)-anchored protein with two variably occupied N-glycosylation sites. In general, GPI-anchor and N-glycosylation direct proteins to apical membranes in polarized cells whereas the majority of mouse PrP^C is found in basolateral membranes in polarized Madin-Darby canine kidney (MDCK) cells. In this study we have mutated the first, the second, and both N-glycosylation sites of PrP^C and also replaced the GPI-anchor of PrP^C by the Thy-1 GPI-anchor in order to investigate the role of these signals in sorting of PrP^C in MDCK cells. Cell surface biotinylation experiments and confocal microscopy showed that lack of one N-linked oligosaccharide leads to loss of polarized sorting of PrP^C. Exchange of the PrP^C GPI-anchor for the one of Thy-1 redirects PrP^C to the apical membrane. In conclusion, both N-glycosylation and GPI-anchor act on polarized sorting of PrP^C, with the GPI-anchor being dominant over N-glycans.

3.1689 Restoration of IFNγR Subunit Assembly, IFNγ Signaling and Parasite Clearance in Leishmania donovani Infected Macrophages: Role of Membrane Cholesterol
Sen, S., Roy, K., Mukherjee, S., Mukhopadhyay, R. and Roym, S.
*PloS Pathogens, **7(9)**, e1002229 (2011)*

Despite the presence of significant levels of systemic Interferon gamma (IFNγ), the host protective cytokine, Kala-azar patients display high parasite load with downregulated IFNγ signaling in *Leishmania donovani* (LD) infected macrophages (LD-MØs); the cause of such aberrant phenomenon is unknown. Here we reveal for the first time the mechanistic basis of impaired IFNγ signaling in parasitized murine macrophages. Our study clearly shows that in LD-MØs IFNγ receptor (IFNγR) expression and their ligand-affinity remained unaltered. The intracellular parasites did not pose any generalized defect in LD-MØs as IL-10 mediated signal transducer and activator of transcription 3 (STAT3) phosphorylation remained unaltered with respect to normal. Previously, we showed that LD-MØs are more fluid than normal MØs due to quenching of membrane cholesterol. The decreased rigidity in LD-MØs was not due to parasite derived lipophosphoglycan (LPG) because purified LPG failed to alter fluidity in normal MØs. IFNγR subunit 1 (IFNγR1) and subunit 2 (IFNγR2) colocalize in raft upon IFNγ stimulation of normal MØs, but this was absent in LD-MØs. Oddly enough, such association of IFNγR1 and IFNγR2 could be restored upon liposomal delivery of cholesterol as evident from the fluorescence resonance energy transfer (FRET) experiment and co-immunoprecipitation studies. Furthermore, liposomal cholesterol treatment together with IFNγ allowed reassociation of signaling assembly (phospho-JAK1, JAK2 and STAT1) in LD-MØs, appropriate signaling, and subsequent parasite killing. This effect was cholesterol specific because cholesterol analogue 4-cholestene-3-one failed to restore the response. The presence of cholesterol binding motifs [(L/V)-$X_{1–5}$-Y-$X_{1–5}$-(R/K)] in the transmembrane domain of IFNγR1 was also noted. The interaction of peptides representing this motif of IFNγR1 was studied with cholesterol-liposome and analogue-liposome with difference of two orders of magnitude in respective affinity (K_D: 4.27×10^{-9} M versus 2.69×10^{-7} M). These observations reinforce the importance of cholesterol in the regulation of function of IFNγR1 proteins. This study clearly demonstrates that during its intracellular life-cycle LD perturbs IFNγR1 and IFNγR2 assembly and subsequent ligand driven signaling by quenching MØ membrane cholesterol.

3.1690 Network Clustering Revealed the Systemic Alterations of Mitochondrial Protein Expression
Jeon, J., Jeong, J.H., Baek, J-H., Koo, H-J., Park, W-H., Yang, J-S., Yu, M-H., Kim, S. and Pak, Y.K.
*PloS Computational Biol., **7(6)**, e1002093 (2011)*

The mitochondrial protein repertoire varies depending on the cellular state. Protein component modifications caused by mitochondrial DNA (*mt*DNA) depletion are related to a wide range of human diseases; however, little is known about how nuclear-encoded mitochondrial proteins (*mt* proteome) changes under such dysfunctional states. In this study, we investigated the systemic alterations of *mt*DNA-depleted (ρ^0) mitochondria by using network analysis of gene expression data. By modularizing the quantified proteomics data into protein functional networks, systemic properties of mitochondrial dysfunction were analyzed. We discovered that up-regulated and down-regulated proteins were organized into two predominant subnetworks that exhibited distinct biological processes. The down-regulated network modules are involved in typical mitochondrial functions, while up-regulated proteins are responsible for *mt*DNA repair and regulation of *mt* protein expression and transport. Furthermore, comparisons of proteome and transcriptome data revealed that ρ^0 cells attempted to compensate for *mt*DNA depletion by modulating the coordinated expression/transport of *mt* proteins. Our results

demonstrate that *mt* protein composition changed to remodel the functional organization of mitochondrial protein networks in response to dysfunctional cellular states. Human *mt* protein functional networks provide a framework for understanding how cells respond to mitochondrial dysfunctions.

3.1691 Mutations associated with Charcot–Marie–Tooth disease cause SIMPLE protein mislocalization and degradation by the proteasome and aggresome–autophagy pathways
Lee, S.M., Olzmann, J.A., Chin, L-S. and Li, L.
*J. Cell Sci., **124(19)**, 3319-3331 (2011)*

Mutations in *SIMPLE* cause an autosomal dominant, demyelinating form of peripheral neuropathy termed Charcot–Marie–Tooth disease type 1C (CMT1C), but the pathogenic mechanisms of these mutations remain unknown. Here, we report that SIMPLE is an early endosomal membrane protein that is highly expressed in the peripheral nerves and Schwann cells. Our analysis has identified a transmembrane domain (TMD) embedded within the cysteine-rich (C-rich) region that anchors SIMPLE to the membrane, and suggests that SIMPLE is a post-translationally inserted, C-tail-anchored membrane protein. We found that CMT1C-linked pathogenic mutations are clustered within or around the TMD of SIMPLE and that these mutations cause mislocalization of SIMPLE from the early endosome membrane to the cytosol. The CMT1C-associated SIMPLE mutant proteins are unstable and prone to aggregation, and they are selectively degraded by both the proteasome and aggresome–autophagy pathways. Our findings suggest that *SIMPLE* mutations cause CMT1C peripheral neuropathy by a combination of loss-of-function and toxic gain-of-function mechanisms, and highlight the importance of both the proteasome and autophagy pathways in the clearance of CMT1C-associated mutant SIMPLE proteins.

3.1692 Lgl1 Activation of Rab10 Promotes Axonal Membrane Trafficking Underlying Neuronal Polarization
Wang, T., Liu, Y., Xu, X-H., Deng, C-Y., Wu, K-Y., Zhu, J., Fu, X-Q., He, M. and Luo, Z-G.
*Developmental Cell, **21(3)**, 431-444 (2011)*

Directed membrane trafficking is believed to be crucial for axon development during neuronal morphogenesis. However, the underlying mechanisms are poorly understood. Here, we report a role of Lgl1, the mammalian homolog of *Drosophila* tumor suppressor Lethal giant larvae, in controlling membrane trafficking underlying axonal growth. We find that Lgl1 is associated with plasmalemmal precursor vesicles and enriched in developing axons. Lgl1 upregulation promoted axonal growth, whereas downregulation attenuated it as well as directional membrane insertion. Interestingly, Lgl1 interacted with and activated Rab10, a small GTPase that mediates membrane protein trafficking, by releasing GDP dissociation inhibitor (GDI) from Rab10. Furthermore, Rab10 lies downstream of Lgl1 in axon development and directional membrane insertion. Finally, both Lgl1 and Rab10 are required for neocortical neuronal polarization in vivo. Thus, the Lgl1 regulation of Rab10 stimulates the trafficking of membrane precursor vesicles, whose fusion with the plasmalemma is crucial for axonal growth.

3.1693 C2 Domain-Containing Phosphoprotein CDP138 Regulates GLUT4 Insertion into the Plasma Membrane
Xie, X., et al
*Cell Metabolism, **14(3)**, 378-389 (2011)*

The protein kinase B_β (Akt2) pathway is known to mediate insulin-stimulated glucose transport through increasing glucose transporter GLUT4 translocation from intracellular stores to the plasma membrane (PM). Combining quantitative phosphoproteomics with RNAi-based functional analyses, we show that a previously uncharacterized 138 kDa C2 domain-containing phosphoprotein (CDP138) is a substrate for Akt2, and is required for optimal insulin-stimulated glucose transport, GLUT4 translocation, and fusion of GLUT4 vesicles with the PM in live adipocytes. The purified C2 domain is capable of binding Ca^{2+} and lipid membranes. CDP138 mutants lacking the Ca^{2+}-binding sites in the C2 domain or Akt2 phosphorylation site S197 inhibit insulin-stimulated GLUT4 insertion into the PM, a rate-limiting step of GLUT4 translocation. Interestingly, CDP138 is dynamically associated with the PM and GLUT4-containing vesicles in response to insulin stimulation. Together, these results suggest that CDP138 is a key molecule linking the Akt2 pathway to the regulation of GLUT4 vesicle-PM fusion.

3.1694 The Mechanism of Tail-Anchored Protein Insertion into the ER Membrane
Wang, F., Whynot, A., Tung, M., and Denic, V.
*Mol. Cell., **43(5)**, 738-750 (2011)*

Tail-anchored (TA) proteins access the secretory pathway via posttranslational insertion of their C-terminal transmembrane domain into the endoplasmic reticulum (ER). Get3 is an ATPase that delivers TA proteins to the ER by interacting with the Get1-Get2 transmembrane complex, but how Get3's nucleotide cycle drives TA protein insertion remains unclear. Here, we establish that nucleotide binding to Get3 promotes Get3-TA protein complex formation by recruiting Get3 to a chaperone that hands over TA proteins to Get3. Biochemical reconstitution and mutagenesis reveal that the Get1-Get2 complex comprises the minimal TA protein insertion machinery with functionally critical cytosolic regions. By engineering a soluble heterodimer of Get1-Get2 cytosolic domains, we uncover the mechanism of TA protein release from Get3: Get2 tethers Get3-TA protein complexes into proximity with the ATPase-dependent, substrate-releasing activity of Get1. Lastly, we show that ATP enhances Get3 dissociation from the membrane, thus freeing Get1-Get2 for new rounds of substrate insertion.

3.1695 The Chlamydia Protease CPAF Regulates Host and Bacterial Proteins to Maintain Pathogen Vacuole Integrity and Promote Virulence
Jorgensen, I., Bednar, M.M., Amin, V., Davis, B.K., Ting, J.P.Y., McCafferty, D.G. and Valdvia, R.H.
*Cell Host & Microbe, **10(1)**, 21-32 (2011)*

The obligate intracellular bacterial pathogen *Chlamydia trachomatis* injects numerous effector proteins into the epithelial cell cytoplasm to manipulate host functions important for bacterial survival. In addition, the bacterium secretes a serine protease, *c*hlamydial *p*rotease-like *a*ctivity *f*actor (CPAF). Although several CPAF targets are reported, the significance of CPAF-mediated proteolysis is unclear due to the lack of specific CPAF inhibitors and the diversity of host targets. We report that CPAF also targets chlamydial effectors secreted early during the establishment of the pathogen-containing vacuole ("inclusion"). We designed a cell-permeable CPAF-specific inhibitory peptide and used it to determine that CPAF prevents superinfection by degrading early *Chlamydia* effectors translocated during entry into a preinfected cell. Prolonged CPAF inhibition leads to loss of inclusion integrity and caspase-1-dependent death of infected epithelial cells. Thus, CPAF functions in niche protection, inclusion integrity and pathogen survival, making the development of CPAF-specific protease inhibitors an attractive antichlamydial therapeutic strategy.

3.1696 Exosomes: immune properties and potential clinical implementations
Chaput, N. and Thery, C.
*Semin. Immunopathol, **33**, 419-440 (2011)*

To communicate, cells are known to release in their environment proteins which bind to receptors on surrounding cells. But cells also secrete more complex structures, called membrane vesicles, composed of a lipid bilayer with inserted transmembrane proteins, enclosing an internal content of hydrophilic components. Exosomes represent a specific subclass of such secreted membrane vesicles, which, despite having been described more than 20 years ago by two groups studying reticulocyte maturation, have only recently received attention from the scientific community. This renewed interest originated first from the description of exosome secretion by antigen-presenting cells, suggesting a potential role in immune responses, and very recently by the identification of the presence of RNA (both messenger and microRNA) in exosomes, suggesting a potential transfer of genetic information between cells. In this review, we will describe the conclusions of 20 years of studies on the immune properties of exosomes and the most recent advances on their roles and potential uses as markers or as therapeutic tools during pathologies, especially in cancer.

3.1697 Caveolin-1 in cytokine-induced enhancement of intracellular Ca2+ in human airway smooth muscle
Sathish, V., Abcejo, A.J., VanOosten, S.K., Thompson, M.A., Prakash, Y.S. and pabelick, C.M.
*Am. J. Physiol. Lung Cell Mol. Physiol., **301(4)**, L607-L614 (2011)*

Diseases such as asthma are characterized by airway hyperresponsiveness. Enhanced airway smooth muscle (ASM) intracellular Ca^{2+} ($[Ca^{2+}]_i$) response to agonist stimulation leading to increased airway constriction has been suggested to contribute to airway hyperresponsiveness. Caveolae are flask-shaped plasma membrane invaginations that express the scaffolding protein caveolin and contain multiple proteins important in $[Ca^{2+}]_i$ signaling (e.g., agonist receptors, ion channels). We recently demonstrated that caveolae and caveolin-1 are important in $[Ca^{2+}]_i$ regulation in human ASM. Proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-13 modulate $[Ca^{2+}]_i$ in ASM. We hypothesized that cytokine upregulation of caveolar signaling in ASM contributes to enhanced agonist-induced $[Ca^{2+}]_i$ in

inflammation. Enzymatically dissociated human ASM cells were exposed to medium (control), 20 ng/ml TNF-α, or 50 ng/ml IL-13 for 24 h. Caveolae-enriched membrane fractions displayed substantial increase in caveolin-1 and -2 expressions by TNF-α and IL-13. Transfection with caveolin-1-mRed DNA substantially accelerated and increased plasma membrane caveolin-1 expression by TNF-α and to a lesser extent by IL-13. Caveolin-1 enhancement was inhibited by nuclear factor-κB and mitogen-activated protein kinase inhibitors. In fura 2-loaded ASM cells, $[Ca^{2+}]_i$ responses to 1 μM ACh, 10 μM histamine, or 10 nM bradykinin were all exaggerated by TNF-α as well as IL-13 exposure. However, disruption of caveolae using caveolin-1 suppression via small-interfering RNA resulted in significant blunting of agonist-induced $[Ca^{2+}]_i$ responses of vehicle and TNF-α-exposed cells. These functional data were correlated to the presence of $TNFR_1$ receptor (but not the IL-4/IL-13 receptor) within caveolae. Overall, these results indicate that caveolin-1 plays an important role in airway inflammation by modulating the effect of specific cytokines on $[Ca^{2+}]_i$.

3.1698 A Nine Amino Acid Domain Is Essential for Mutant Prion Protein Toxicity

Westergard, L., Turnbaugh, J.A. and Harris, D.A.
J. Neurosci., ***31(39)****, 14005-14017 (2011)*

Transgenic mice expressing prion protein (PrP) molecules with several different internal deletions display spontaneous neurodegenerative phenotypes that can be dose-dependently suppressed by coexpression of wild-type PrP. Each of these deletions, including the largest one (Δ32–134), retains 9 aa immediately following the signal peptide cleavage site (residues 23–31; KKRPKPGGW). These residues have been implicated in several biological functions of PrP, including endocytic trafficking and binding of glycosaminoglycans. We report here on our experiments to test the role of this domain in the toxicity of deleted forms of PrP. We find that transgenic mice expressing Δ23–134 PrP display no clinical symptoms or neuropathology, in contrast to mice expressing Δ32–134 PrP, suggesting that residues 23–31 are essential for the toxic phenotype. Using a newly developed cell culture assay, we narrow the essential region to amino acids 23–26, and we show that mutant PrP toxicity is not related to the role of the N-terminal residues in endocytosis or binding to endogenous glycosaminoglycans. However, we find that mutant PrP toxicity is potently inhibited by application of exogenous glycosaminoglycans, suggesting that the latter molecules block an essential interaction between the N terminus of PrP and a membrane-associated target site. Our results demonstrate that a short segment containing positively charged amino acids at the N terminus of PrP plays an essential role in mediating PrP-related neurotoxicity. This finding identifies a protein domain that may serve as a drug target for amelioration of prion neurotoxicity.

3.1699 Invasion of Cryptococcus neoformans into Human Brain Microvascular Endothelial Cells Is Mediated through the Lipid Rafts-Endocytic Pathway via the Dual Specificity Tyrosine Phosphorylation-regulated Kinase 3 (DYRK3)

Huang, S-H., Long, M., Wu, C-H., Kwon-Chung, K.J., Chang, Y.C., Chi, F., Lee, S. and Jong, A.
J. Biol. Chem., ***286(40)****,34761-34769 (2011)*

Cryptococcus neoformans is a neurotropic fungal pathogen, which provokes the onset of devastating meningoencephalitis. We used human brain microvascular endothelial cells (HBMEC) as the *in vitro* model to investigate how *C. neoformans* traverses across the blood-brain barrier. In this study, we present several lines of evidence indicating that *C. neoformans* invasion is mediated through the endocytic pathway via lipid rafts. Human CD44 molecules from lipid rafts can directly interact with hyaluronic acid, the *C. neoformans* ligand. Bikunin, which perturbs CD44 function in the lipid raft, can block *C. neoformans* adhesion and invasion of HBMEC. The lipid raft marker, ganglioside GM1, co-localizes with CD44 on the plasma membrane, and *C. neoformans* cells can adhere to the host cell in areas where GM1 is enriched. These findings suggest that *C. neoformans* entry takes place on the lipid rafts. Upon *C. neoformans* engagement, GM1 is internalized through vesicular structures to the nuclear membrane. This endocytic redistribution process is abolished by cytochalasin D, nocodazole, or anti-DYRK3 (dual specificity tyrosine-phosphorylation-regulated kinase 3) siRNA. Concomitantly, the knockdown of DYRK3 significantly reduces *C. neoformans* invasion across the HBMEC monolayer *in vitro*. Our data demonstrate that the lipid raft-dependent endocytosis process mediates *C. neoformans* internalization into HBMEC and that the CD44 protein of the hosts, cytoskeleton, and intracellular kinase-DYRK3 are involved in this process.

3.1700 Elicitation of Epithelial Cell-Derived Immune Effectors by Outer Membrane Vesicles of Nontypeable Haemophilus influenza

Sharpe, S.W., Kuehn, M.J. and Mason, K.M.

Infect. Immun., ***79(11)***, *4361-4369 (2011)*

Outer membrane vesicles (OMVs) are produced by all Gram-negative microorganisms studied to date. The contributions of OMVs to biological processes are diverse and include mediation of bacterial stress responses, selective packaging and secretion of virulence determinants, modulation of the host immune response, and contributions to biofilm formation and stability. First characterized as transformasomes in *Haemophilus*, these membranous blebs facilitate transfer of DNA among bacteria. Nontypeable *Haemophilus influenzae* (NTHI), an opportunistic pathogen of the upper and lower respiratory tracts, produces OMVs *in vivo*, but there is a paucity of information regarding both the composition and role of OMVs during NTHI colonization and pathogenesis. We demonstrated that purified NTHI vesicles are 20 to 200 nm in diameter and contain DNA, adhesin P5, IgA endopeptidase, serine protease, and heme utilization protein, suggesting a multifaceted role in virulence. NTHI OMVs can bind to human pharyngeal epithelial cells, resulting in a time- and temperature-dependent aggregation on the host cell surface, with subsequent internalization. OMVs colocalize with the endocytosis protein caveolin, indicating that internalization is mediated by caveolae, which are cholesterol-rich lipid raft domains. Upon interaction with epithelial cells, NTHI OMVs stimulate significant release of the immunomodulatory cytokine interleukin-8 (IL-8) as well as the antimicrobial peptide LL-37. Thus, we demonstrated that NTHI OMVs contain virulence-associated proteins that dynamically interact with and invade host epithelial cells. Beyond their ability to mediate DNA transfer in *Haemophilus*, OMV stimulation of host immunomodulatory cytokine and antimicrobial peptide release supports a dynamic role for vesiculation in NTHI pathogenesis and clinically relevant disease progression.

3.1701 EBV-gp350 Confers B-Cell Tropism to Tailored Exosomes and Is a Neo-Antigen in Normal and Malignant B Cells—A New Option for the Treatment of B-CLL

Ruiss, R., Jochum, S., Mocikat, R., Hammerschmidt, W. and Zeidler, R.

PloS One, ***6(10)***, *e25294 (2011)*

gp350, the major envelope protein of Epstein-Barr-Virus, confers B-cell tropism to the virus by interacting with the B lineage marker CD21. Here we utilize gp350 to generate tailored exosomes with an identical tropism. These exosomes can be used for the targeted co-transfer of functional proteins to normal and malignant human B cells. We demonstrate here the co-transfer of functional CD154 protein on tailored gp350+ exosomes to malignant B blasts from patients with B chronic lymphocytic leukemia (B-CLL), rendering B blasts immunogenic to tumor-reactive autologous T cells. Intriguingly, engulfment of gp350+ exosomes by B-CLL cells and presentation of gp350-derived peptides also re-stimulated EBV-specific T cells and redirected the strong antiviral cellular immune response in patients to leukemic B cells. In essence, we show that gp350 alone confers B-cell tropism to exosomes and that these exosomes can be further engineered to simultaneously trigger virus- and tumor-specific immune responses. The simultaneous exploitation of gp350 as a tropism molecule for tailored exosomes and as a neo-antigen in malignant B cells provides a novel attractive strategy for immunotherapy of B-CLL and other B-cell malignancies.

3.1702 The Endosomal Na+/H+ Exchanger Contributes to Multivesicular Body Formation by Regulating the Recruitment of ESCRT-0 Vps27p to the Endosomal Membrane

Mitsui, K., Koshimura, Y., Yoshikawa, Y., matsushita, M. and Kanazawa, H.

J. Biol. Chem., ***286(43)***, *37625-37638 (2011)*

Multivesicular bodies (MVBs) are late endosomal compartments containing luminal vesicles (MVB vesicles) that are formed by inward budding of the endosomal membrane. In budding yeast, MVBs are an important cellular mechanism for the transport of membrane proteins to the vacuolar lumen. This process requires a class E subset of vacuolar protein sorting (*VPS*) genes. *VPS44* (allelic to *NHX1*) encodes an endosome-localized Na^+/H^+ exchanger. The function of the *VPS44* exchanger in the context of vacuolar protein transport is largely unknown. Using a cell-free MVB formation assay system, we demonstrated that Nhx1p is required for the efficient formation of MVB vesicles in the late endosome. The recruitment of Vps27p, a class E Vps protein, to the endosomal membrane was dependent on Nhx1p activity and was enhanced by an acidic pH at the endosomal surface. Taken together, we propose that Nhx1p contributes to MVB formation by the recruitment of Vps27p to the endosomal membrane, possibly through Nhx1p antiporter activity.

3.1703 c-Cbl-Mediated Selective Virus-Receptor Translocations into Lipid Rafts Regulate Productive Kaposi's Sarcoma-Associated Herpesvirus Infection in Endothelial Cells
Chakraborty, S., Veettil, M.V., Sadagopan, S., Paudel, N. and Chandran, B.
J. Virol., ***85(23)***, *12410-12430 (2011)*

During target cell entry and infection, many enveloped and nonenveloped viruses utilize cell surface receptors that translocate into lipid rafts (LRs). However, the mechanism behind this translocation is not known. Kaposi's sarcoma-associated herpesvirus (KSHV) interacts with the human microvascular dermal endothelial (HMVEC-d) cell surface heparan sulfate (HS), integrins α3β1, αVβ3, and αVβ5, and the amino acid transporter x-CT protein and enters via c-Cbl-bleb-mediated macropinocytosis (Veettil et al., J. Virol. **82:**12126-12144, 2008; Veettil et al., PLoS Pathog. **6:**e1001238, 2010). Here we have demonstrated that very early during infection (1 min postinfection), c-Cbl induced the selective translocation of KSHV into the LR along with the α3β1, αVβ3, and x-CT receptors but not αVβ5. Activated c-Cbl localized with LRs at the junctional base of macropinocytic blebs. LR-translocated α3β1 and αVβ3 were monoubiquitinated, leading to productive macropinocytic entry, whereas non-LR-associated αVβ5 was polyubiquitinated, leading to clathrin entry that was targeted to lysosomes. c-Cbl knockdown blocked the macropinocytosis and receptor translocation and diverted KSHV to a clathrin-lysosomal noninfectious pathway. Similar results were also seen by LR disruption with MβCD. These studies provide the first evidence that c-Cbl regulates selective KSHV-α3β1, -αVβ3, and -x-CT receptor translocations into the LRs and differential ubiquitination of receptors which are critical determinants of the macropinocytic entry route and productive infection of KSHV. Our studies suggest that interventions targeting c-Cbl and LRs are potential avenues to block KSHV infection of endothelial cells.

3.1704 Endothelial progenitor cell–dependent angiogenesis requires localization of the full-length form of uPAR in caveolae
Margheri, F., Chilla, A., Laurenzana, A., Serrati, S., Mazzanti, B., Saccardi, R., Santosuosso, S., Danza, G., Sturli, N., Rosati, F., Magnelli, L., Papucci, L., Calorini, L., Bianchini, F., Del Rosso, M. and Fibbi, G.
Blood, ***118(13)***, *3743-3755 (2011)*

Endothelial urokinase-type plasminogen activator receptor (uPAR) is thought to provide a regulatory mechanism in angiogenesis. Here we studied the proangiogenic role of uPAR in endothelial colony-forming cells (ECFCs), a cell population identified in human umbilical blood that embodies all of the properties of an endothelial progenitor cell matched with a high proliferative rate. By using caveolae-disrupting agents and by caveolin-1 silencing, we have shown that the angiogenic properties of ECFCs depend on caveolae integrity and on the presence of full-length uPAR in such specialized membrane invaginations. Inhibition of *uPAR* expression by antisense oligonucleotides promoted caveolae disruption, suggesting that uPAR is an inducer of caveolae organization. Vascular endothelial growth factor (VEGF) promoted accumulation of uPAR in ECFC caveolae in its undegraded form. We also demonstrated that VEGF-dependent ERK phosphorylation required integrity of caveolae as well as caveolar uPAR expression. VEGF activity depends on inhibition of ECFC MMP12 production, which results in impairment of MMP12-dependent uPAR truncation. Further, *MMP12* overexpression in ECFC inhibited vascularization in vitro and in vivo. Our data suggest that intratumor homing of ECFCs suitably engineered to overexpress *MMP12* could have the chance to control uPAR-dependent activities required for tumor angiogenesis and malignant cells spreading.

3.1705 Analysis of Detergent-free Lipid Rafts isolated from a CD4+ T cell line: Interaction with antigen presenting cells promotes coalescing of lipid rafts
Kennedy, C., Nelson, M.D. and Bamezai, A.K.
Cell Communication and Signaling, ***9****, 31-xxx (2011)*

Background: Lipid rafts present on the plasma membrane play an important role in spatiotemporal regulation of cell signaling. Physical and chemical characterization of lipid raft size and assessment of their composition before, and after cell stimulation will aid in developing a clear understanding of their regulatory role in cell signaling. We have used visual and biochemical methods and approaches for examining individual and lipid raft sub-populations isolated from a mouse CD4+ T cell line in the absence of detergents.
Results: Detergent-free rafts were analyzed before and after their interaction with antigen presenting cells. We provide evidence that the average diameter of lipid rafts isolated from unstimulated T cells, in the absence of detergents, is less than 100 nm. Lipid rafts on CD4+ T cell

membranes coalesce to form larger structures, after interacting with antigen presenting cells even in the absence of a foreign antigen.
Conclusions: Findings presented here indicate that lipid raft coalescence occurs during cellular interactions prior to sensing a foreign antigen

3.1706 Lysosomal accumulation of Trk protein in brain of GM1-gangliosidosis mouse and its restoration by chemical chaperone

Takamura, A., Higaki, K., Ninomiya, H., Takai, T., matsuda, J., Iida, M., Ohno, K., Suzuki, Y. and nanba, E.
J. Neurochem., ***118(3)***, *399-406 (2011)*

G_{M1}-gangliosidosis is a fatal neurodegenerative disorder caused by deficiency of lysosomal acid β-galactosidase (β-gal). Accumulation of its substrate ganglioside G_{M1} (G_{M1}) in lysosomes and other parts of the cell leads to progressive neurodegeneration, but underlying mechanisms remain unclear. Previous studies demonstrated an essential role for interaction of G_{M1} with tropomyosin receptor kinase (Trk) receptors in neuronal growth, survival and differentiation. In this study we demonstrate accumulation of G_{M1} in the cell-surface rafts and lysosomes of the β-gal knockout (β-gal−/−) mouse brain association with accumulation of Trk receptors and enhancement of its downstream signaling. Immunofluorescence and subcellular fractionation analysis revealed accumulation of Trk receptors in the late endosomes/lysosomes of the β-gal−/− mouse brain and their association with ubiquitin and p62. Administration of a chemical chaperone to β-gal−/− mouse expressing human mutant R201C protein resulted in a marked reduction of intracellular storage of G_{M1} and phosphorylated Trk. These findings indicate that G_{M1} accumulation in rafts causes activation of Trk signaling, which may participate in the pathogenesis of G_{M1}-gangliosidosis.

3.1707 Neuronal glycoprotein M6a induces filopodia formation via association with cholesterol-rich lipid rafts

Scorticati, C., Formoso, K. and Frasch, A.C.
J. Neurochem., ***119(3)***, *521-531 (2011)*

A neuronal integral membrane glycoprotein M6a has been suggested to be involved in a number of biological processes, including neuronal remodeling and differentiation, trafficking of mu-opioid receptors, and Ca^{2+} transportation. Moreover, pathological situations such as chronic stress in animals and depression in humans have been associated with alterations in M6a sequence and expression. The mechanism of action of M6a is essentially unknown. In this work, we analyze the relevance of M6a distribution in plasma membrane, namely its lipid microdomain targeting, for its biological function in filopodia formation. We demonstrate that M6a is localized in membrane microdomains compatible with lipid rafts in cultured rat hippocampal neurons. Removal of cholesterol from neuronal membranes with methyl-β-cyclodextrin decreases M6a-induced filopodia formation, an effect that is reversed by the addition of cholesterol. Inhibition of Src kinases and MAPK prevents filopodia formation in M6a-over-expressing neurons. Src-deficient SYF cells over-expressing M6a fail to promote filopodia formation. Taken together, our findings reveal that the association of M6a with lipid rafts is important for its role in filopodia formation and Src and MAPK kinases participate in M6a signal propagation.

3.1708 Endocytosis is essential for dynamic translocation of a syntaxin 1 orthologue during fission yeast meiosis

Kashiwazaki, J., Yamasaki, Y., Itadani, A., Teraguchi, E., Maeda, Y., Shimoda, C. and Nakamura, T.
Mol. Biol. Cell, ***22(19)***, *3658-3670 (2011)*

Syntaxin is a component of the target soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor complex, which is responsible for fusion of membrane vesicles at the target membrane. The fission yeast syntaxin 1 orthologue Psy1 is essential for both vegetative growth and spore formation. During meiosis, Psy1 disappears from the plasma membrane (PM) and dramatically relocalizes on the nascent forespore membrane, which becomes the PM of the spore. Here we report the molecular details and biological significance of Psy1 relocalization. We find that, immediately after meiosis I, Psy1 is selectively internalized by endocytosis. In addition, a meiosis-specific signal induced by the transcription factor Mei4 seems to trigger this internalization. The internalization of many PM proteins is facilitated coincident with the initiation of meiosis, whereas Pma1, a P-type ATPase, persists on the PM even during the progression of meiosis II. Ergosterol on the PM is also important for the internalization of PM proteins in general during meiosis. We consider that during meiosis in *Schizosaccharomyces pombe* cells, the

characteristics of endocytosis change, thereby facilitating internalization of Psy1 and accomplishing sporulation.

3.1709 Retrolinkin cooperates with endophilin A1 to mediate BDNF–TrkB early endocytic trafficking and signaling from early endosomes
Fu, X., Yang, Y., Xu, C., Niu, Y., Chen, T., Zhou, Q. and Liu, J-J.
*Mol. Biol. Cell, **22(19)**, 3684-3698 (2011)*

Brain-derived neurotrophic factor (BDNF) binds to its cell surface receptor TrkB to regulate differentiation, development, synaptic plasticity, and functional maintenance of neuronal cells. Binding of BDNF triggers TrkB dimerization and autophosphorylation, which provides docking sites for adaptor proteins to recruit and activate downstream signaling molecules. The molecular mechanisms underlying BDNF–TrkB endocytic trafficking crucial for spatiotemporal control of signaling pathways remain to be elucidated. Here we show that retrolinkin, a transmembrane protein, interacts with endophilin A1 and mediates BDNF-activated TrkB (pTrk) trafficking and signaling in CNS neurons. We find that activated TrkB colocalizes and interacts with the early endosome marker APPL1. Both retrolinkin and endophilin A1 are required for BDNF-induced dendrite development and acute extracellular signal-regulated kinase activation from early endosomes. Suppression of retrolinkin expression not only blocks BDNF-triggered TrkB internalization, but also prevents recruitment of endophilin A1 to pTrk vesicles trafficking through APPL1-positive endosomes. These findings reveal a novel mechanism for BDNF–TrkB to regulate signaling both in time and space through a specific membrane trafficking pathway.

3.1710 Distinct Autophagosomal-Lysosomal Fusion Mechanism Revealed by Thapsigargin-Induced Autophagy Arrest
Ganley, I.G., Wong, P-M., Gammoth, N. and Jiang, X.
*Mol. Cell, **42(6)**, 731-743 (2011)*

Autophagy, a catabolic pathway that delivers cellular components to lysosomes for degradation, can be activated by stressful conditions such as nutrient starvation and endoplasmic reticulum (ER) stress. We report that thapsigargin, an ER stressor widely used to induce autophagy, in fact blocks autophagy. Thapsigargin does not affect autophagosome formation but leads to accumulation of mature autophagosomes by blocking autophagosome fusion with the endocytic system. Strikingly, thapsigargin has no effect on endocytosis-mediated degradation of epidermal growth factor receptor. Molecularly, while both Rab7 and Vps16 are essential regulatory components for endocytic fusion with lysosomes, we found that Rab7 but not Vps16 is required for complete autophagy flux, and that thapsigargin blocks recruitment of Rab7 to autophagosomes. Therefore, autophagosomal-lysosomal fusion must be governed by a distinct molecular mechanism compared to general endocytic fusion.

3.1711 Conserved Proline-Rich Region of Ebola Virus Matrix Protein VP40 Is Essential for Plasma Membrane Targeting and Virus-Like Particle Release
Reynard, O., Nemirov, K., page, A., mateo, M., Raoul, H., Weissenhorn, W. and Volchkov, V.
*J. Infectious Dis., **204**, Suppl.3 , S884-S889 (2011)*

The matrix protein VP40 is essential for Ebola virus (EBOV) and Marburg virus assembly and budding at the plasma membrane. In this study we have investigated the effect of single amino acid substitutions in a conserved proline-rich region of the EBOV VP40 located in the carboxy-terminal part of the protein. We demonstrate that substitutions within this region result in an alteration of intracellular VP40 localization and also cause a reduction or a complete block of virus-like particle budding, a benchmark of VP40 function. Furthermore, some mutated VP40s revealed an enhanced binding with cellular Sec24C, a part of the coat protein complex II (COPII) vesicular transport system. Analysis of the 3-dimensional structure of VP40 revealed the spatial proximity of the proline-rich region and an earlier identified site of interaction with Sec24C, thus allowing us to hypothesize that the altered intracellular localization of the VP40 mutants is a consequence of defects in their interaction with COPII-mediated vesicular transport.

3.1712 A new specialization in astrocytes: Glutamate- and ammonia-induced nuclear size changes
Yang, C.Z., Li, H.L., Zhou, Y., Chai, R.C., Zhao, R., Dong, Y., Xu, Z.Y., Lau, L.T., Yingge, Z., Teng, J., Chen, J and Yu, A.C.H.
*J. Neurosci. Res., **89(12)**, 2041-2051 (2011)*

We observed nuclear swelling in glutamate (Glu)-treated astrocytes that was concomitant with but independent of astrocytic cell swelling. We confirmed Glu-induced nuclear swelling with nuclei isolated from astrocytes. Ammonia is metabolically related to Glu and could induce a nuclear swelling in intact astrocytes but shrinkage in isolated nuclei. Other compounds such as glutamine, aspartate, taurine, glycine, and ATP did not cause any nuclear swelling in isolated nuclei of astrocytes. Surprisingly, Glu and ammonia did not induce nuclear swelling in microglia, C6, HEK 293, or Hep G2 cell lines in cultures and their isolated nuclei. The Glu- and ammonia-induced nuclear size changes appear to be a specific response of astrocytes to these two closely related metabolic compounds

3.1713 Ndel1, Nudel (Noodle): Flexible in the cell?
Chansard, M., Hong, J-H., park, Y-U., park, S.K., and Nguyen, M.D.
Cytoskeleton, ***68(10)****, 540-554 (2011)*

Nuclear distribution element-like 1 (Ndel1 or Nudel) was firstly described as a regulator of the cytoskeleton in microtubule and intermediate filament dynamics and microtubule-based transport. Emerging evidence indicates that Ndel1 also serves as a docking platform for signaling proteins and modulates enzymatic activities (kinase, ATPase, oligopeptidase, GTPase). Through these structural and signaling functions, Ndel1 plays a role in diverse cellular processes (e.g., mitosis, neurogenesis, neurite outgrowth, and neuronal migration). Furthermore, Ndel1 is linked to the etiology of various mental illnesses and neurodegenerative disorders. In the present review, we summarize the physiological and pathological functions associated with Ndel1. We further advance the concept that Ndel1 interfaces GTPases-mediated processes (endocytosis, vesicles morphogenesis/signaling) and cytoskeletal dynamics to impact cell signaling and behaviors. This putative mechanism may affect cellular functionalities and may contribute to shed light into the causes of devastating human diseases.

3.1714 Defective photoreceptor phagocytosis in a mouse model of enhanced S-cone syndrome causes progressive retinal degeneration
Mustafi, D., Kevany, B.M., Genoud, C., Okano, K., Cideciyan, A.V., Sumaroka, A., Roman, A.J., Jacobson, S.G., Engel, A., Adams, M.D. and Palczewski, K.
FASEB J., ***25(9)****, 3157-3176 (2011)*

Enhanced S-cone syndrome (ESCS), featuring an excess number of S cones, manifests as a progressive retinal degeneration that leads to blindness. Here, through optical imaging, we identified an abnormal interface between photoreceptors and the retinal pigment epithelium (RPE) in 9 patients with ESCS. The neural retina leucine zipper transcription factor-knockout ($Nrl^{-/-}$) mouse model demonstrates many phenotypic features of human ESCS, including unstable S-cone-positive photoreceptors. Using massively parallel RNA sequencing, we identified 6203 differentially expressed transcripts between wild-type (*Wt*) and $Nrl^{-/-}$ mouse retinas, with 6 highly significant differentially expressed genes of the Pax, Notch, and Wnt canonical pathways. Changes were also obvious in expression of 30 genes involved in the visual cycle and 3 key genes in photoreceptor phagocytosis. Novel high-resolution (100 nm) imaging and reconstruction of $Nrl^{-/-}$ retinas revealed an abnormal packing of photoreceptors that contributed to buildup of photoreceptor deposits. Furthermore, lack of phagosomes in the RPE layer of $Nrl^{-/-}$ retina revealed impairment in phagocytosis. Cultured RPE cells from *Wt* and $Nrl^{-/-}$ mice illustrated that the phagocytotic defect was attributable to the aberrant interface between ESCS photoreceptors and the RPE. Overcoming the retinal phagocytosis defect could arrest the progressive degenerative component of this disease.—Mustafi, D., Kevany, B. M., Genoud, C., Okano, K., Cideciyan, A. V., Sumaroka, A., Roman, A. J., Jacobson, S. G. Engel, A., Adams, M. D., Palczewski, K. Defective photoreceptor phagocytosis in a mouse model of enhanced S-cone syndrome causes progressive retinal degeneration.

3.1715 FATP4 contributes as an enzyme to the basal and insulin-mediated fatty acid uptake of C2C12 muscle cells
Digel, M., Staffer, S., Ehehalt, F., Stremmel, W., Ehehalt, R. and Füllekrug, J.
Am. J. Physiol. Endocrinol. Metab., ***301(5)****, E785-E796 (2011)*

The function of membrane proteins in long-chain fatty acid transport is controversial. The acyl-CoA synthetase fatty acid transport protein-4 (FATP4) has been suggested to facilitate fatty acid uptake indirectly by its enzymatic activity, or directly by transport across the plasma membrane. Here, we investigated the function of FATP4 in basal and insulin mediated fatty acid uptake in C_2C_{12} muscle cells, a model system relevant for fatty acid metabolism. Stable expression of exogenous FATP4 resulted in a twofold higher fatty acyl-CoA synthetase activity, and cellular uptake of oleate was enhanced similarly.

Kinetic analysis demonstrated that FATP4 allowed the cells to reach apparent saturation of fatty acid uptake at a twofold higher level compared with control. Short-term treatment with insulin increased fatty acid uptake in line with previous reports. Surprisingly, insulin increased the acyl-CoA synthetase activity of C_2C_{12} cells within minutes. This effect was sensitive to inhibition of insulin signaling by wortmannin. Affinity purified FATP4 prepared from insulin-treated cells showed an enhanced enzyme activity, suggesting it constitutes a novel target of short-term metabolic regulation by insulin. This offers a new mechanistic explanation for the concomitantly observed enhanced fatty acid uptake. FATP4 was colocalized to the endoplasmic reticulum by double immunofluorescence and subcellular fractionation, clearly distinct from the plasma membrane. Importantly, neither differentiation into myotubes nor insulin treatment changed the localization of FATP4. We conclude that FATP4 functions by its intrinsic enzymatic activity. This is in line with the concept that intracellular metabolism plays a significant role in cellular fatty acid uptake.

3.1716 Quantitative subproteomic analysis of age-related changes in mouse liver peroxisomes by iTRAQ LC–MS/MS
Amelina, H., Sjödin, M.O.D., Bergquist, J. and Cristobal, S.
*J. Chromatography B, **879(10)**, 3393-3400 (2011)*

Aging is a complex multifactorial phenomenon, which is believed to result from the accumulation of cellular damage to biological macromolecules. Peroxisomes recently emerged as another important source of reactive oxygen species (ROS) production in addition to mitochondria. However, the role of these organelles in the process of aging is still not clear. The aim of this study was to characterize the changes in protein expression profiles of young (10 weeks old) versus old (18 months old) mouse liver peroxisome-enriched fractions. We have applied shotgun proteomic approach based on liquid chromatography and tandem mass spectrometry (LC–MS/MS) combined with iTRAQ (isobaric tags for relative and absolute quantitation) labeling that allows comparative quantitative multiplex analysis. Our analysis led to identification and quantification of 150 proteins, 8 out of which were differentially expressed between two age groups at a statistically significant level ($p < 0.05$), with folds ranging from 1.2 to 4.1. These proteins involved in peroxisomal β-oxidation, detoxification of xenobiotics and production of ROS. Noteworthy, differences in liver proteome have been observed between as well as within different age groups. In conclusion, our subproteomic quantitative study suggests that mouse liver proteome is sufficiently maintained until certain age.

3.1717 In vitro nuclear egress of herpes simplex virus type 1 capsids
Remillard-Labrosse, G. and Lippe, R.
*Methods, **55(2)**, 153-159 (2011)*

During their life cycles, viruses typically undergo many transport events throughout the cell. These events depend on a variety of both viral and host proteins and are often not fully understood. Such studies are often complicated by asynchronous infections and the concurrent presence of various viral intermediates in the cells, making it difficult to molecularly define each step. In the case of the herpes simplex virus type 1, the etiological agent of cold sores and many other illnesses, the viral particles undergo an intricate series of transport steps during its life cycle. Upon entry by fusion with a cellular membrane, they travel to the host cell nucleus where the virus replicates and assembles new viral particles. These particles then travel across the two nuclear envelopes and transit through the trans-Golgi network before finally being transported to and released at the cell surface. Though viral components and some host proteins modulating these numerous transport events have been identified, the details of these processes remain to be elucidated. To specifically address how the virus escapes the nucleus, we set up an *in vitro* model that reproduces the unconventional route used by herpes simplex type 1 virus to leave nuclei. This has not only allowed us to clarify the route of capsid egress of the virus but is now useful to define it at the molecular level.

3.1718 GLUT2 Accumulation in Enterocyte Apical and Intracellular Membranes: A Study in Morbidly Obese Human Subjects and ob/ob and High Fat–Fed Mice
Ait-Omar, A. et al
*Diabetes, **60**, 2598-2607 (2011)*

OBJECTIVE In healthy rodents, intestinal sugar absorption in response to sugar-rich meals and insulin is regulated by GLUT2 in enterocyte plasma membranes. Loss of insulin action maintains apical GLUT2 location. In human enterocytes, apical GLUT2 location has not been reported but may be revealed under conditions of insulin resistance.

RESEARCH DESIGN AND METHODS Subcellular location of GLUT2 in jejunal enterocytes was analyzed by confocal and electron microscopy imaging and Western blot in 62 well-phenotyped morbidly obese subjects and 7 lean human subjects. GLUT2 locations were assayed in *ob*/*ob* and *ob*/+ mice receiving oral metformin or in high-fat low-carbohydrate diet–fed C57Bl/6 mice. Glucose absorption and secretion were respectively estimated by oral glucose tolerance test and secretion of [U-^{14}C]-3-*O*-methyl glucose into lumen.
RESULTS In human enterocytes, GLUT2 was consistently located in basolateral membranes. Apical GLUT2 location was absent in lean subjects but was observed in 76% of obese subjects and correlated with insulin resistance and glycemia. In addition, intracellular accumulation of GLUT2 with early endosome antigen 1 (EEA1) was associated with reduced MGAT4a activity (glycosylation) in 39% of obese subjects on a low-carbohydrate/high-fat diet. Mice on a low-carbohydrate/high-fat diet for 12 months also exhibited endosomal GLUT2 accumulation and reduced glucose absorption. In *ob*/*ob* mice, metformin promoted apical GLUT2 and improved glucose homeostasis. Apical GLUT2 in fasting hyperglycemic *ob*/*ob* mice tripled glucose release into intestinal lumen.
CONCLUSIONS In morbidly obese insulin-resistant subjects, GLUT2 was accumulated in apical and/or endosomal membranes of enterocytes. Functionally, apical GLUT2 favored and endosomal GLUT2 reduced glucose transepithelial exchanges. Thus, altered GLUT2 locations in enterocytes are a sign of intestinal adaptations to human metabolic pathology.

3.1719 Motor neuron impairment mediated by a sumoylated fragment of the glial glutamate transporter EAAT2

Foran, E., Bogush, A., Goffredo, M., Roncaglia, P., Gustincich, S., Pasinell, P. and Trotti, D.
Glia, 59(11), 1719-1731 (2011)

Dysregulation of glutamate handling ensuing downregulation of expression and activity levels of the astroglial glutamate transporter EAAT2 is implicated in excitotoxic degeneration of motor neurons in amyotrophic lateral sclerosis (ALS). We previously reported that EAAT2 (a.k.a. GLT-1) is cleaved by caspase-3 at its cytosolic carboxy-terminus domain. This cleavage results in impaired glutamate transport activity and generates a proteolytic fragment (CTE) that we found to be post-translationally conjugated by SUMO1. We show here that this sumoylated CTE fragment accumulates in the nucleus of spinal cord astrocytes of the SOD1-G93A mouse model of ALS at symptomatic stages of disease. Astrocytic expression of CTE, artificially tagged with SUMO1 (CTE-SUMO1) to mimic the native sumoylated fragment, recapitulates the nuclear accumulation pattern of the endogenous EAAT2-derived proteolytic fragment. Moreover, in a co-culture binary system, expression of CTE-SUMO1 in spinal cord astrocytes initiates extrinsic toxicity by inducing caspase-3 activation in motor neuron-derived NSC-34 cells or axonal growth impairment in primary motor neurons. Interestingly, prolonged nuclear accumulation of CTE-SUMO1 is intrinsically toxic to spinal cord astrocytes, although this gliotoxic effect of CTE-SUMO1 occurs later than the indirect, noncell autonomous toxic effect on motor neurons. As more evidence on the implication of SUMO substrates in neurodegenerative diseases emerges, our observations strongly suggest that the nuclear accumulation in spinal cord astrocytes of a sumoylated proteolytic fragment of the astroglial glutamate transporter EAAT2 could participate to the pathogenesis of ALS and suggest a novel, unconventional role for EAAT2 in motor neuron degeneration.

3.1720 Characterizing the Escherichia coli O157:H7 Proteome Including Protein Associations with Higher Order Assemblies

Pieper, R., Zhang, Q., Clark, D.J., Hunag, S-T., Suh, M-J., Braisted, J.C., Payne, S.H., Fleischmann, R.D., Peterson, S.N. and Tzipori, S.
*PloS One, **6(10)**, e26554 (2011)*

Background
The recent outbreak of severe infections with Shiga toxin (Stx) producing *Escherichia coli* (STEC) serotype O104:H4 highlights the need to understand horizontal gene transfer among *E. coli* strains, identify novel virulence factors and elucidate their pathogenesis. Quantitative shotgun proteomics can contribute to such objectives, allowing insights into the part of the genome translated into proteins and the connectivity of biochemical pathways and higher order assemblies of proteins at the subcellular level.
Methodology/Principal Findings
We examined protein profiles in cell lysate fractions of STEC strain 86-24 (serotype O157:H7), following growth in cell culture or bacterial isolation from intestines of infected piglets, in the context of functionally and structurally characterized biochemical pathways of *E. coli*. Protein solubilization in the presence of

Triton X-100, EDTA and high salt was followed by size exclusion chromatography into the approximate M_r ranges greater than 280 kDa, 280-80 kDa and 80-10 kDa. Peptide mixtures resulting from these and the insoluble fraction were analyzed by quantitative 2D-LC-nESI-MS/MS. Of the 2521 proteins identified at a 1% false discovery rate, representing 47% of all predicted *E. coli* O157:H7 gene products, the majority of integral membrane proteins were enriched in the high M_r fraction. Hundreds of proteins were enriched in a M_r range higher than that predicted for a monomer supporting their participation in protein complexes. The insoluble STEC fraction revealed enrichment of aggregation-prone proteins, including many that are part of large structure/function entities such as the ribosome, cytoskeleton and O-antigen biosynthesis cluster.
Significance
Nearly all *E. coli* O157:H7 proteins encoded by prophage regions were expressed at low abundance levels or not detected. Comparative quantitative analyses of proteins from distinct cell lysate fractions allowed us to associate uncharacterized proteins with membrane attachment, potential participation in stable protein complexes, and susceptibility to aggregation as part of larger structural assemblies.

3.1721 Differential Palmit(e)oylation of Wnt1 on C93 and S224 Residues Has Overlapping and Distinct Consequences
Galli, L.M. and Burrus, L.W.
*PloS One, **6(10)**, e26636 (2011)*

Though the mechanisms by which cytosolic/intracellular proteins are regulated by the post-translational addition of palmitate adducts is well understood, little is known about how this lipid modification affects secreted ligands, such as Wnts. Here we use mutational analysis to show that differential modification of the two known palmit(e)oylated residues of Wnt1, C93 and S224, has both overlapping and distinct consequences. Though the relative roles of each residue are similar with respect to stability and secretion, two distinct biological assays in L cells show that modification of C93 primarily modulates signaling via a ß-catenin independent pathway while S224 is crucial for ß-catenin dependent signaling. In addition, pharmacological inhibition of Porcupine (Porcn), an upstream regulator of Wnt, by IWP1, specifically inhibited ß-catenin dependent signaling. Consistent with these observations, mapping of amino acids in peptide domains containing C93 and S224 demonstrate that acylation of C93 is likely to be Porcn-independent while that of S224 is Porcn-dependent. Cumulatively, our data strongly suggest that C93 and S224 are modified by distinct enzymes and that the differential modification of these sites has the potential to influence Wnt signaling pathway choice.

3.1722 Rescue of Calcineurin Aα−/− Mice Reveals a Novel Role for the α Isoform in the Salivary Gland
Reddy, R.N., Pena, J.A., Roberts, B.R., Williams, S.R., Price, S.R. and Gooch, J.L.
*Am. J. Pathol., **178(4)**, 1605-1613 (2011)*

Calcineurin is an important signal transduction mediator in T cells, neurons, the heart, and kidneys. Recent evidence points to unique actions of the two main isoforms of the catalytic subunit. Although the β isoform is required for T-cell development, α is important in the brain and kidney. In addition, mice lacking α but not β suffer from failure to thrive and early mortality. The purpose of this study was to identify the cause of postnatal death of calcineurin α null ($CnA\alpha^{-/-}$) mice and to determine the mechanism of α activity that contributes to the phenotype. $CnA\alpha^{-/-}$ mice and wild-type littermate controls were fed a modified diet and then salivary gland function and histology were examined. *In vitro* studies were performed to identify the mechanism of α action. Data show that calcineurin is required for normal submandibular gland function and secretion of digestive enzymes. Loss of α does not impair nuclear factor of activated T-cell activity or expression but results in impaired protein trafficking downstream of the inositol trisphosphate receptor. These findings show a novel function of calcineurin in digestion and protein trafficking. Significantly, these data also provide a mechanism to rescue to adulthood a valuable animal model of calcineurin inhibitor-mediated neuronal and renal toxicities.

3.1723 HAT4, a Golgi Apparatus-Anchored B-Type Histone Acetyltransferase, Acetylates Free Histone H4 and Facilitates Chromatin Assembly
Yang, X., Yu, W., Shi, L., Sun, L., Linag, J., Yi, X., Li, Q., Zhang, Y., Yang, F., Han, X., Zhang, D., Yang, J., Yao, Z. and Shang, Y.
*Mol. Cell, **44(1)**, 39-50 (2011)*

Histone acetyltransferases (HATs) are an essential regulatory component in chromatin biology. Unlike A-type HATs, which are found in the nucleus and utilize nucleosomal histones as substrates and thus primarily function in transcriptional regulation, B-type HATs have been characterized as cytoplasmic

enzymes that catalyze the acetylation of free histones. Here, we report on a member of the GCN5-related N-acetyltransferase superfamily and another B-type HAT, HAT4. Interestingly, HAT4 is localized in the Golgi apparatus and displays a substrate preference for lysine residues of free histone H4, including H4K79 and H4K91, that reside in the globular domain of H4. Significantly, HAT4 depletion impaired nucleosome assembly, inhibited cell proliferation, sensitized cells to DNA damage, and induced cell apoptosis. Our data indicate that HAT4 is an important player in the organization and function of the genome and may contribute to the diversity and complexity of higher eukaryotic organisms.

3.1724 The Unconventional Role of Acid Sphingomyelinase in Regulation of Retinal Microangiopathy in Diabetic Human and Animal Models

Opreanu, M., Tikhonenko, M., Bozack, S., Lydic, T.A., Reid, G.E., McSorley, K.M., Sochacki, A., Perez, G.I., Esselman, W.J., Kern, T., Kolesnick, R., Grant, M.B. and Busik, J.V.
Diabetes, ***60***, *2370-2378 (2011)*

OBJECTIVE Acid sphingomyelinase (ASM) is an important early responder in inflammatory cytokine signaling. The role of ASM in retinal vascular inflammation and vessel loss associated with diabetic retinopathy is not known and represents the goal of this study.
RESEARCH DESIGN AND METHODS Protein and gene expression profiles were determined by quantitative RT-PCR and Western blot. ASM activity was determined using Amplex Red sphingomyelinase assay. Caveolar lipid composition was analyzed by nano-electrospray ionization tandem mass spectrometry. Streptozotocin-induced diabetes and retinal ischemia-reperfusion models were used in in vivo studies.
RESULTS We identify endothelial caveolae-associated ASM as an essential component in mediating inflammation and vascular pathology in in vivo and in vitro models of diabetic retinopathy. Human retinal endothelial cells (HREC), in contrast with glial and epithelial cells, express the plasma membrane form of ASM that overlaps with caveolin-1. Treatment of HREC with docosahexaenoic acid (DHA) specifically reduces expression of the caveolae-associated ASM, prevents a tumor necrosis factor-α–induced increase in the ceramide-to-sphingomyelin ratio in the caveolae, and inhibits cytokine-induced inflammatory signaling. ASM is expressed in both vascular and neuroretina; however, only vascular ASM is specifically increased in the retinas of animal models at the vasodegenerative phase of diabetic retinopathy. The absence of ASM in $ASM^{-/-}$ mice or inhibition of ASM activity by DHA prevents acellular capillary formation.
CONCLUSIONS This is the first study demonstrating activation of ASM in the retinal vasculature of diabetic retinopathy animal models. Inhibition of ASM could be further explored as a potential therapeutic strategy in treating diabetic retinopathy.

3.1725 Outer Membrane Vesicles Induce Immune Responses to Virulence Proteins and Protect against Colonization by Enterotoxigenic Escherichia coli

Roy, K., Hamilton, D.J., Munson, G.P. and Fleckenstein, J.M.
Clin. Vacc. Immunol., ***18(11)***, *1803-1808 (2011)*

Enterotoxigenic Escherichia coli (ETEC) strains are a heterogeneous group of pathogens that produce heat-labile (LT) and/or heat-stable (ST) enterotoxins. Collectively, these pathogens are responsible for hundreds of thousands of deaths annually in developing countries, particularly in children under the age of 5 years. The heterogeneity of previously investigated molecular targets and the lack of complete sustained protection afforded by antitoxin immunity have impeded progress to date toward a broadly protective vaccine. Many pathogens, including ETEC, have the capacity to form outer membrane vesicles (OMV), which often contain one or more virulence proteins. Prompted by recent studies that identified several immunogenic virulence proteins in outer membrane vesicles of ETEC, we sought to examine the immunogenicity and protective efficacy of these structures in a murine model of infection. Here we demonstrate that immunization with OMV impairs ETEC colonization of the small intestine and stimulates antibodies that recognize the heat-labile toxin and two additional putative virulence proteins, the EtpA adhesin and CexE. Similar to earlier studies with EtpA, vaccination with LT alone also inhibited intestinal colonization. Together, these findings suggest that OMV could be exploited to deliver protective antigens relevant to development of ETEC vaccines.

3.1726 Screening and Optimization of Ligand Conjugates for Lysosomal Targeting

Meerovich, I., Hoshkaryev, A., Thekkedath, R. and Torchilin, V.P.
Bioconjugate Chem., ***22(11)***, *2271-2282 (2011)*

The use of lysosome-targeted liposomes may significantly improve the delivery of therapeutic enzymes and chaperones into lysosomes for the treatment of lysosomal storage disorders. The aim of this research was to synthesize new potentially lysosomotropic ligands on a base of Neutral Red and rhodamine B and to study their ability to enhance specific lysosomal delivery of surface-modified liposomes loaded with a model compound, fluorescein isothiocyanate-dextran (FD). The delivery of these liposomes and their content to lysosomes in HeLa cells was investigated by confocal immunofluorescent microscopy, subcellular fractionation, and flow cytometry. Confocal microscopy demonstrated that liposomes modified with derivatives of rhodamine B provide a good rate of colocalization with the specific lysosomal markers. The comparison of fluorescence of FD in lysosomes isolated by subcellular fractionation also showed that the efficiency of lysosomal delivery of the liposomal load by liposomes modified with some of synthesized ligands was significantly higher compared to that with plain liposomes. These results were additionally confirmed by flow cytometry of the intact cells treated with liposomes loaded with 5-dodecanoylaminofluorescein di-β-d-galactopyranoside, a specific substrate for the intralysosomal β-galactosidase, using a number of cell lines, including macrophages with induced phenotype of lysosomal enzyme deficiency; two of the synthesized ligands—rhodamine B DSPE-PEG_{2k}-amide and 6-(3-(DSPE-PEG_{2k})-thioureido) rhodamine B—demonstrated enhanced lysosomal delivery, in some cases, higher than that for commercially available rhodamine B octadecyl ester, with the best results (the enhancement of the lysosomal delivery up to 75% greater in comparison to plain liposomes) shown for the cells with induced lysosomal enzyme deficiency phenotype. Use of liposomes modified with rhodamine B derivatives may be advantageous for the development of drug delivery systems for the treatment of lysosome-associated disorders.

3.1727 Retinal Degeneration 3 (RD3) Protein Inhibits Catalytic Activity of Retinal Membrane Guanylyl Cyclase (RetGC) and Its Stimulation by Activating Proteins
Peshenko, I.V., Olshevskaya, EW.V., Azadi, S., Molday, L.L., Molday, R.S. and Dizhoor, A.M:
Biochemistry, ***50(44)***, *9511-9519 (2011)*

Retinal membrane guanylyl cyclase (RetGC) in the outer segments of vertebrate photoreceptors is controlled by guanylyl cyclase activating proteins (GCAPs), responding to light-dependent changes of the intracellular Ca^{2+} concentrations. We present evidence that a different RetGC binding protein, *r*etinal *d*egeneration *3* protein (RD3), is a high-affinity allosteric modulator of the cyclase which inhibits RetGC activity at submicromolar concentrations. It suppresses the basal activity of RetGC in the absence of GCAPs in a noncompetitive manner, and it inhibits the GCAP-stimulated RetGC at low intracellular Ca^{2+} levels. RD3 opposes the allosteric activation of the cyclase by GCAP but does not significantly change Ca^{2+} sensitivity of the GCAP-dependent regulation. We have tested a number of mutations in RD3 implicated in human retinal degenerative disorders and have found that several mutations prevent the stable expression of RD3 in HEK293 cells and decrease the affinity of RD3 for RetGC1. The RD3 mutant lacking the carboxy-terminal half of the protein and associated with Leber congenital amaurosis type 12 (LCA12) is unable to suppress the activity of the RetGC1/GCAP complex. Furthermore, the inhibitory activity of the G57V mutant implicated in cone–rod degeneration is strongly reduced. Our results suggest that inhibition of RetGC by RD3 may be utilized by photoreceptors to block RetGC activity during its maturation and/or incorporation into the photoreceptor outer segment rather than participate in dynamic regulation of the cyclase by Ca^{2+} and GCAPs.

3.1728 Translation initiation factors and active sites of protein synthesis co-localize at the leading edge of migrating fibroblasts
Willett, M., Brocard, M., Davide, A. and Morley, S.J.
Biochem. J., ***438***, *217-227 (2011)*

Cell migration is a highly controlled essential cellular process, often dysregulated in tumour cells, dynamically controlled by the architecture of the cell. Studies involving cellular fractionation and microarray profiling have previously identified functionally distinct mRNA populations specific to cellular organelles and architectural compartments. However, the interaction between the translational machinery itself and cellular structures is relatively unexplored. To help understand the role for the compartmentalization and localized protein synthesis in cell migration, we have used scanning confocal microscopy, immunofluorescence and a novel ribopuromycylation method to visualize translating ribosomes. In the present study we show that eIFs (eukaryotic initiation factors) localize to the leading edge of migrating MRC5 fibroblasts in a process dependent on TGN (*trans*-Golgi network) to plasma membrane vesicle transport. We show that eIF4E and eIF4GI are associated with the Golgi apparatus and

membrane microdomains, and that a proportion of these proteins co-localize to sites of active translation at the leading edge of migrating cells.

3.1729 Lipid Raft Localization of EGFR Alters the Response of Cancer Cells to the EGFR Tyrosine Kinase Inhibitor Gefitinib

Irwin, M.E., Mueller, K.L., Bohin, N., Ge, Y. and Boerner, J.L.
J. Cell. Physiol., ***226***, *2316-2328 (2011)*

Epidermal growth factor receptor (EGFR) is overexpressed in many cancer types including_30% of breast cancers. Several small molecule tyrosine kinase inhibitors (TKIs) targeting EGFR have shown clinical efficacy in lung and colon cancers, but no benefit has been noted in breast cancer. Thirteen EGFR expressing breast cancer cell lines were analyzed for response to EGFR TKIs. Seven were found to be EGFR TKI resistant; while shRNA knockdown of EGFR determined that four of these cell lines retained the requirement of EGFR protein expression for growth. Interestingly, EGFR localized to plasma membrane lipid rafts in all four of these EGFR TKI-resistant cell lines, as determined by biochemical raft isolation and immunofluorescence. When lipid rafts were depleted of cholesterol using lovastatin, all four cell lines were sensitized to EGFR TKIs. In fact, the effects of the cholesterol biosynthesis inhibitor and gefitinib were synergistic. While gefitinib effectively abrogated phosphorylation of Akt- and mitogen-activated protein kinase in an EGFR TKI-sensitive cell line, phosphorylation of Akt persisted in two EGFR TKI-resistant cell lines, however, this phosphorylation was abrogated by lovastatin treatment. Thus, we have shown that lipid raft localization of EGFR correlates with resistance to EGFR TKI-induced growth inhibition and pharmacological depletion of cholesterol from lipid rafts decreases this resistance in breast cancer cell lines. Furthermore, we have presented evidence to suggest that when EGFR localizes to lipid rafts, these rafts provide a platform to facilitate activation of Akt signalling in the absence of EGFR kinase activity.

3.1730 Activating Transcription Factor 6 Limits Intracellular Accumulation of Mutant α1-Antitrypsin Z and Mitochondrial Damage in Hepatoma Cells

Smith, S.E., Granell, S., Salcedo-Sicilia, L., Baldini, G., Egea, G., Teckman, J.H. and Baldini, G.
J. Biol. Chem., ***286(48)***, *41563-41577 (2011)*

α_1-Antitrypsin is a serine protease inhibitor secreted by hepatocytes. A variant of α_1-antitrypsin with an E342K (Z) mutation (ATZ) has propensity to form polymers, is retained in the endoplasmic reticulum (ER), is degraded by both ER-associated degradation and autophagy, and causes hepatocyte loss. Constant features in hepatocytes of PiZZ individuals and in PiZ transgenic mice expressing ATZ are the formation of membrane-limited globular inclusions containing ATZ and mitochondrial damage. Expression of ATZ in the liver does not induce the unfolded protein response (UPR), a protective mechanism aimed to maintain ER homeostasis in the face of an increased load of proteins. Here we found that in hepatoma cells the ER E3 ligase HRD1 functioned to degrade most of the ATZ before globular inclusions are formed. Activation of the activating transcription factor 6 (ATF6) branch of the UPR by expression of spliced ATF6(1–373) decreased intracellular accumulation of ATZ and the formation of globular inclusions by a pathway that required HRD1 and the proteasome. Expression of ATF6(1–373) in ATZ-expressing hepatoma cells did not induce autophagy and increased the level of the proapoptotic factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) but did not lead to apoptotic DNA fragmentation. Expression of ATF6(1–373) did not cause inhibition of protein synthesis and prevented mitochondrial damage induced by ATZ expression. It was concluded that activation of the ATF6 pathway of the UPR limits ATZ-dependent cell toxicity by selectively promoting ER-associated degradation of ATZ and is thereby a potential target to prevent hepatocyte loss in addition to autophagy-enhancing drugs.

3.1731 C323 of SR-BI is required for SR-BI-mediated HDL binding and cholesteryl ester uptake

Guo, L., Chen, M., Song, Z., Daugherty, A. and Li, X-A.
J. Lipid Res., ***52***, *2272-2278 (2011)*

Scavenger receptor BI (SR-BI) is an HDL receptor. It binds HDL and mediates the uptake of cholesteryl ester from HDL. Early studies have pointed out that the extracellular domain of SR-BI is critical for SR-BI-mediated cholesteryl ester uptake. However, the extracellular loop of SR-BI is large: it contains 403 amino acids. The HDL binding site and the modulation of SR-BI-mediated cholesteryl ester uptake remain to be identified. In this study, using C323G mutant SR-BI, we showed that C323G mutant SR-BI lost its HDL binding and cholesteryl ester uptake activity, indicating that the highly conserved C323 is required

for SR-BI-mediated HDL binding and cholesteryl ester uptake. Using a blocking antibody against C323 region, we demonstrated that C323 is directly involved in HDL binding and likely an HDL binding site. Using C323G mutant transgenic mouse model, we further demonstrated that C323 of SR-BI is required for regulating plasma cholesterol levels in vivo. Using redox reagents, we showed that physiological relevant levels of H_2O_2 upregulated the SR-BI-mediated cholesteryl ester uptake activity by 65%, whereas GSH or DTT significantly downregulated SR-BI-mediated cholesteryl ester uptake activity by 45%. C323 of SR-BI is critical for SR-BI-mediated HDL binding and cholesteryl ester uptake, and changes in redox status may be a regulatory factor modulating SR-BI-mediated cholesterol transport.

3.1732 Site-directed mutagenesis of human cytosolic sulfotransferase (SULT) 2B1b to phospho-mimetic Ser348Asp results in an isoform with increased catalytic activity
Salman, E.D., He, D., Runge-Morris, M., Kocarek, T.A. and Falany, C.N.
J. Steroid Biochem. Mol. Biol., ***127***, *315-323 (2011)*

Human SULT2B1b is distinct from other SULT isoforms due to the presence of unique amino (N)- and carboxy (C)-terminal peptides. Using site-directed mutagenesis, it was determined that phosphorylation of Ser348 was associated with nuclear localization. To investigate the effects of this phosphorylation of Ser348 on activity and cellular localization, an in silico molecular mimic was generated by mutating Ser348 to an Asp. The Asp residue mimics the shape and charge of a phospho-Ser and homology models of SULT2B1b-phospho-S348 and SULT2B1b-S348D suggest a similar significant structural rearrangement in the C-terminal peptide. To evaluate the functional consequences of this post-translational modification and predicted rearrangement, 6His-SULT2B1b-S348D was synthesized, expressed, purified and characterized. The 6His-SULT2B1b-S348D has a specific activity for DHEA sulfation ten-fold higher than recombinant 6His-SULT2B1b (209.6 and 21.8 pmol min−1 mg−1, respectively). Similar to native SULT2B1b, gel filtration chromatography showed SULT2B1b-S348D was enzymatically active as a homodimer. Stability assays comparing SULT2B1b and SUL2B1b-S348 demonstrated that SULT2B1b is 60% less thermostable than SULT2B1b-348D. The increased stability and sulfation activity allowed for better characterization of the sulfation kinetics for putative substrates as well as the determination of dissociation constants that were difficult to obtain with wild-type (WT) 6His-SULT2B1b. The KDs for DHEA and PAPS binding to 6His-SULT2B1b-S348D were 650 ± 7 nM and 265 ± 4 nM, respectively, whereas KDs for binding of substrates to the WT enzyme could not be determined. Characterization of the molecular mimic SULT2B1b-S348D provides a better understanding for the role of the unique structure of SULT2B1b and its effect on sulfation activity, and has allowed for improved kinetic characterization of the SULT2B1b enzyme.

3.1733 Thyroid-specific knockout of the tumor suppressor mitogen-inducible gene 6 activates epidermal growth factor receptor signaling pathways and suppresses nuclear factor-κB activity
Lin, C-I., Barletta, J.A., Nehs, M.A., Morris, Z.S., Donner, D.B., Whang, E.E., Jeong, J-w., Kimura, S., Moore, F.D. and Ruan, D.T.
Surgery, ***150(1)***, *295-302 (2011)*

Background
Mitogen-inducible gene 6 (Mig-6) is a putative tumor suppressor gene and prognostic biomarker in papillary thyroid cancer. We hypothesized that Mig-6 knockout would activate pro-oncogenic signaling in mouse thyrocytes.
Methods
We performed a thyroid-specific knockout using the Cre/*loxP* recombinase system.
Results
Four knockout and 4 control mouse thyroids were harvested at 2 months of age. Immunoblotting confirmed Mig-6 ablation in knockout mice thyrocytes. Epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK) phosphorylation levels were increased in Mig-6 knockout compared to wild-type mice. Total EGFR levels were similar in knockout and wild-type mice. However, EGFR was absent in the caveolae-containing membrane fraction of knockout mice, indicating that Mig-6 depletion is associated with a change in the membrane distribution of EGFR. Although p65 localized to the nucleus in wild-type mice, it was distributed in both cytoplasm and nucleus in knockouts, suggesting that Mig-6 loss decreases p65 activity.
Conclusion
Our results confirm the feasibility of targeted, thyroid-specific gene knockout as a strategy for studying the relevance of specific genes in thyroid oncogenesis. We suggest that the loss of Mig-6 alters the membrane distribution of EGFR, which may limit receptor degradation and activate this oncogenic signaling pathway.

3.1734 Biochemical and Molecular Mechanisms of Folate Transport in Rat Pancreas; Interference with Ethanol Ingestion

Wani, N.A., Nada, R. and Kaur, J.
*PloS One, **6(12)**, e28599 (2011)*

Folic acid is an essential nutrient that is required for one-carbon biosynthetic processes and for methylation of biomolecules**.** Deficiency of this micronutrient leads to disturbances in normal physiology of cell. Chronic alcoholism is well known to be associated with folate deficiency which is due, in part to folate malabsorption. The present study deals with the mechanistic insights of reduced folate absorption in pancreas during chronic alcoholism. Male Wistar rats were fed 1 g/kg body weight/day ethanol (20% solution) orally for 3 months and the mechanisms of alcohol associated reduced folate uptake was studied in pancreas. The folate transport system in the pancreatic plasma membrane (PPM) was found to be acidic pH dependent one. The transporters proton coupled folate transporter (PCFT) and reduced folate carrier (RFC) are involved in folate uptake across PPM. The folate transporters were found to be associated with lipid raft microdomain of the PPM. Ethanol ingestion decreased the folate transport by reducing the levels of folate transporter molecules in lipid rafts at the PPM. The decreased transport efficiency of the PPM was reflected as reduced folate levels in pancreas. The chronic ethanol ingestion led to decreased pancreatic folate uptake. The decreased levels of PCFT and RFC expression in rat PPM were due to decreased association of these proteins with lipid rafts (LR) at the PPM.

3.1735 Nuclear Localization of de Novo Thymidylate Biosynthesis Pathway Is Required to Prevent Uracil Accumulation in DNA

MacFarlane, A.J., Anderson, D.D., Flodby, P., Perry, C.A., Allen, R.H. and Stabler, S.P.
*J. Biol. Chem., **286(51)**, 44015-44022 (2011)*

Uracil accumulates in DNA as a result of impaired folate-dependent *de novo* thymidylate biosynthesis, a pathway composed of the enzymes serine hydroxymethyltransferase (SHMT), thymidylate synthase (TYMS), and dihydrofolate reductase. In G_1, this pathway is present in the cytoplasm and at S phase undergoes small ubiquitin-like modifier-dependent translocation to the nucleus. It is not known whether this pathway functions in the cytoplasm, nucleus, or both *in vivo*. SHMT1 generates 5,10-methylenetetrahydrofolate for *de novo* thymidylate biosynthesis, a limiting step in the pathway, but also tightly binds 5-methyltetrahydrofolate in the cytoplasm, a required cofactor for homocysteine remethylation. Overexpression of SHMT1 in cell cultures inhibits folate-dependent homocysteine remethylation and enhances thymidylate biosynthesis. In this study, the impact of increased *Shmt1* expression on folate-mediated one-carbon metabolism was determined in mice that overexpress the *Shmt1* cDNA (*Shmt1*$^{tg+}$ mice). Compared with wild type mice, *Shmt1*$^{tg+}$ mice exhibited elevated SHMT1 and TYMS protein levels in tissues and evidence for impaired homocysteine remethylation but surprisingly exhibited depressed levels of nuclear SHMT1 and TYMS, lower rates of nuclear *de novo* thymidylate biosynthesis, and a nearly 10-fold increase in uracil content in hepatic nuclear DNA when fed a folate- and choline-deficient diet. These results demonstrate that SHMT1 and TYMS localization to the nucleus is essential to prevent uracil accumulation in nuclear DNA and indicate that SHMT1-mediated nuclear *de novo* thymidylate synthesis is critical for maintaining DNA integrity.

3.1736 Quantitative proteomics reveals metabolic and pathogenic properties of Chlamydia trachomatis developmental forms

Saka, H.A., Thompson, J.W., Chen, Y-S:, Kumar, Y., Dubois, L.G., Moseley, M.A. and Valdivia, R.H.
*Mol. Microbiol., **82(5)**, 1185-1203 (2011)*

Chlamydia trachomatis is an obligate intracellular pathogen responsible for ocular and genital infections of significant public health importance. *C. trachomatis* undergoes a biphasic developmental cycle alternating between two distinct forms: the infectious elementary body (EB), and the replicative but non-infectious reticulate body (RB). The molecular basis for these developmental transitions and the metabolic properties of the EB and RB forms are poorly understood as these bacteria have traditionally been difficult to manipulate through classical genetic approaches. Using two-dimensional liquid chromatography – tandem mass spectrometry (LC/LC-MS/MS) we performed a large-scale, label-free quantitative proteomic analysis of *C. trachomatis* LGV-L2 EB and RB forms. Additionally, we carried out LC-MS/MS to analyse the membranes of the pathogen-containing vacuole (‘inclusion’). We developed a label-free quantification approaches to measure protein abundance in a mixed-proteome background which we applied for EB and RB quantitative analysis. In this manner, we catalogued the relative distribution of > 54% of the predicted

proteins in the *C. trachomatis* LGV-L2 proteome. Proteins required for central metabolism and glucose catabolism were predominant in the EB, whereas proteins associated with protein synthesis, ATP generation and nutrient transport were more abundant in the RB. These findings suggest that the EB is primed for a burst in metabolic activity upon entry, whereas the RB form is geared towards nutrient utilization, a rapid increase in cellular mass, and securing the resources for an impending transition back to the EB form. The most revealing difference between the two forms was the relative deficiency of cytoplasmic factors required for efficient type III secretion (T3S) in the RB stage at 18 h post infection, suggesting a reduced T3S capacity or a low frequency of active T3S apparatus assembled on a 'per organism' basis. Our results show that EB and RB proteomes are streamlined to fulfil their predicted biological functions: maximum infectivity for EBs and replicative capacity for RBs.

3.1737 Effects of brefeldin A-inhibited guanine nucleotide-exchange (BIG) 1 and KANK1 proteins on cell polarity and directed migration during wound healing
Li, C-C., Kuo,, J-C., Waterman, C.M., Kiyama, R., Moss, J. and Vaughan, M.
*PNAS, **108(48)**, 19228-19233 (2011)*

Brefeldin A-inhibited guanine nucleotide-exchange protein (BIG) 1 activates class I ADP ribosylation factors (ARFs) by accelerating the replacement of bound GDP with GTP to initiate recruitment of coat proteins for membrane vesicle formation. Among proteins that interact with BIG1, kinesin family member 21A (KIF21A), a plus-end-directed motor protein, moves cargo away from the microtubule-organizing center (MTOC) on microtubules. Because KANK1, a protein containing N-terminal KN, C-terminal ankyrin-repeat, and intervening coiled-coil domains, has multiple actions in cells and also interacts with KIF21A, we explored a possible interaction between it and BIG1. We obtained evidence for a functional and physical association between these proteins, and found that the effects of BIG1 and KANK1 depletion on cell migration in wound-healing assays were remarkably similar. Treatment of cells with BIG1- or KANK1-specific siRNA interfered significantly with directed cell migration and initial orientation of Golgi/MTOC toward the leading edge, which was not mimicked by KIF21A depletion. Although colocalization of overexpressed KANK1 and endogenous BIG1 in HeLa cells was not clear microscopically, their reciprocal immunoprecipitation (IP) is compatible with the presence of small percentages of each protein in the same complexes. Depletion or overexpression of BIG1 protein appeared not to affect KANK1 distribution. Our data identify actions of both BIG1 and KANK1 in regulating cell polarity during directed migration; these actions are consistent with the presence of both BIG1 and KANK1 in dynamic multimolecular complexes that maintain Golgi/MTOC orientation, differ from those that might contain all three proteins (BIG1, KIF21A, and KANK1), and function in directed transport along microtubules.

3.1738 The Human Immunodeficiency Virus Coat Protein gp120 Promotes Forward Trafficking and Surface Clustering of NMDA Receptors in Membrane Microdomains
Xu, H., Bae, M., Tovar-Romo, L.B., patel, N., Bandaru, V.V.R., Pomerantz, D., Steiner, J.P. and Haughey, N.J.
*J. Neurosci., **31(47)**, 17074-17090 (2011)*

Infection by the human immunodeficiency virus (HIV) can result in debilitating neurological syndromes collectively known as HIV-associated neurocognitive disorders. Although the HIV coat protein gp120 has been identified as a potent neurotoxin that enhances NMDA receptor function, the exact mechanisms for this effect are not known. Here we provide evidence that gp120 activates two separate signaling pathways that converge to enhance NMDA-evoked calcium flux by clustering NMDA receptors in modified membrane microdomains. gp120 enlarged and stabilized the structure of lipid microdomains on dendrites by mechanisms that involved a redox-regulated translocation of a sphingomyelin hydrolase (neutral sphingomyelinase-2) to the plasma membrane. A concurrent pathway was activated that accelerated the forward traffic of NMDA receptors by a PKA-dependent phosphorylation of the NR1 C-terminal serine 897 (masks an ER retention signal), followed by a PKC-dependent phosphorylation of serine 896 (important for surface expression). NMDA receptors were preferentially targeted to synapses and clustered in modified membrane microdomains. In these conditions, NMDA receptors were unable to laterally disperse and did not internalize, even in response to strong agonist induction. Focal NMDA-evoked calcium bursts were enhanced by threefold in these regions. Inhibiting membrane modification or NR1 phosphorylation prevented gp120 from accelerating the surface localization of NMDA receptors. Disrupting the structure of membrane microdomains after gp120 treatments restored the ability of NMDA receptors to disperse and internalize. These findings demonstrate that gp120 contributes to synaptic dysfunction in the setting of HIV infection by interfering with NMDA receptor trafficking.

3.1739 Analysis of RhoA and Rho GEF activity in whole cells and the cell nucleus
Guilluy, C., Dubash, A. and Garcia-mata, R.
*Nature Protocols, **6(12)**, 2050-2060 (2011)*

We have recently shown that a fraction of the total cellular pool of the small GTPase RhoA resides in the nucleus, and that the nuclear guanine nucleotide exchange factor (GEF) Net1 has a role in the regulation of its activity. In this protocol, we describe a method to measure both the activities of the nuclear pools of RhoA and Rho GEFs. This process required the development of a nuclear isolation protocol that is both fast and virtually free of cytosolic and membrane contaminants, as well as a redesign of existing RhoA and Rho GEF activity assays so that they work in nuclear samples. This protocol can be also used for other Rho GTPases and Rho GEFs, which have also been found in the nucleus. Completion of the procedure, including nuclear isolation and RhoA or Rho GEF activity assay, takes 1 h 40 min. We also include details of how to perform a basic assay of whole-cell extracts.

3.1740 A Highly Dynamic ER-Derived Phosphatidylinositol-Synthesizing Organelle Supplies Phosphoinositides to Cellular Membranes
Kim, Y.J., Guzman-Hernandez, M.L. and Balla, T.
*Developmental Cell, **21**, 813-824 (2011)*

Polyphosphoinositides are lipid signaling molecules generated from phosphatidylinositol (PtdIns) with critical roles in vesicular trafficking and signaling. It is poorly understood where PtdIns is located within cells and how it moves around between membranes. Here we identify a hitherto-unrecognized highly mobile membrane compartment as the site of PtdIns synthesis and a likely source of PtdIns of all membranes. We show that the PtdIns-synthesizing enzyme PIS associates with a rapidly moving compartment of ER origin that makes ample contacts with other membranes. In contrast, CDP-diacylglycerol synthases that provide PIS with its substrate reside in the tubular ER. Expression of a PtdInsspecific bacterial PLC generates diacylglycerol also in rapidly moving cytoplasmic objects. We propose a model in which PtdIns is synthesized in a highly mobile lipid distribution platform and is delivered to other membranes during multiple contacts by yet-to-be-defined lipid transfer mechanisms.

3.1741 Saturated Fatty Acids Induce c-Src Clustering within Membrane Subdomains, Leading to JNK Activation
Holzer, R.G., Park, E-J., Li, N., Tran, H., Chen, M., Choi, C., Solinas, G. and Karin, M.
*Cell, **147(1)**, 173-184 (2011)*

Saturated fatty acids (FA) exert adverse health effects and are more likely to cause insulin resistance and type 2 diabetes than unsaturated FA, some of which exert protective and beneficial effects. Saturated FA, but not unsaturated FA, activate Jun N-terminal kinase (JNK), which has been linked to obesity and insulin resistance in mice and humans. However, it is unknown how saturated and unsaturated FA are discriminated. We now demonstrate that saturated FA activate JNK and inhibit insulin signaling through c-Src activation. FA alter the membrane distribution of c-Src, causing it to partition into intracellular membrane subdomains, where it likely becomes activated. Conversely, unsaturated FA with known beneficial effects on glucose metabolism prevent c-Src membrane partitioning and activation, which are dependent on its myristoylation, and block JNK activation. Consumption of a diabetogenic high-fat diet causes the partitioning and activation of c-Src within detergent insoluble membrane subdomains of murine adipocytes.

3.1742 Unique Properties of the ATP-Sensitive K+ Channel in the Mouse Ventricular Cardiac Conduction System
Bao, L., Kefaloyianni, E., Lader, J., Hong, M., Morley, G., Fishman, G.I., Sobie, E.A. and Coetzee, W.A.
*Cirr. Arrhythm. Electrophysiol., **4**, 926-935 (2011)*

Background—The specialized cardiac conduction system (CCS) expresses a unique complement of ion channels that confer a specific electrophysiological profile. ATP-sensitive potassium (K_{ATP}) channels in these myocytes have not been systemically investigated.
Methods and Results—We recorded K_{ATP} channels in isolated CCS myocytes using Cntn2-EGFP reporter mice. The CCS K_{ATP} channels were less sensitive to inhibitory cytosolic ATP compared with ventricular channels and more strongly activated by MgADP. They also had a smaller slope conductance. The 2 types of channels had similar intraburst open and closed times, but the CCS K_{ATP} channel had a prolonged

interburst closed time. CCS K_{ATP} channels were strongly activated by diazoxide and less by levcromakalim, whereas the ventricular K_{ATP} channel had a reverse pharmacological profile. CCS myocytes express elevated levels of Kir6.1 but reduced Kir6.2 and SUR2A mRNA compared with ventricular myocytes (SUR1 expression was negligible). SUR2B mRNA expression was higher in CCS myocytes relative to SUR2A. Canine Purkinje fibers expressed higher levels of Kir6.1 and SUR2B protein relative to the ventricle. Numeric simulation predicts a high sensitivity of the Purkinje action potential to changes in ATP:ADP ratio. Cardiac conduction time was prolonged by low-flow ischemia in isolated, perfused mouse hearts, which was prevented by glibenclamide.
Conclusions—These data imply a differential electrophysiological response (and possible contribution to arrhythmias) of the ventricular CCS to K_{ATP} channel opening during periods of ischemia.

3.1743 Proline-Serine-Threonine Phosphatase-Interacting Protein 2 (PSTPIP2), a Host Membrane-Deforming Protein, Is Critical for Membranous Web Formation in Hepatitis C Virus Replication
Chao, T-C., Su, W-C., Huang, J-Y., Chen, Y-C., Jeng, K-S., Wang, H-D. and Lai, M.M.C.
*J. Virol., **86(3)**, 1739-1749 (2012)*

Hepatitis C virus (HCV) reorganizes intracellular membranes to establish sites of replication. How viral and cellular proteins target, bind, and rearrange specific membranes into the replication factory remains a mystery. We used a lentivirus-based RNA interference (RNAi) screening approach to identify the potential cellular factors that are involved in HCV replication. A protein with membrane-deforming activity, proline-serine-threonine phosphatase-interacting protein 2 (PSTPIP2), was identified as a potential factor. Knockdown of PSTPIP2 in HCV subgenomic replicon-harboring and HCV-infected cells was associated with the reduction of HCV protein and RNA expression. PSTPIP2 was localized predominantly in detergent-resistant membranes (DRMs), which contain the RNA replication complex. PSTPIP2 knockdown caused a significant reduction of the formation of HCV- and NS4B-induced membranous webs. A PSTPIP2 mutant defective in inducing membrane curvature failed to support HCV replication, confirming that the membrane-deforming ability of PSTPIP2 is essential for HCV replication. Taking these results together, we suggest that PSTPIP2 facilitates membrane alterations and is a key player in the formation of the membranous web, which is the site of the HCV replication complex.

3.1744 Cholesterol dependence of Newcastle Disease Virus entry
Martin, J.J., Holguera, J., Sanchez-Felipe, L., Villar, E. and Munoz-Barroso, I.
*Biochem. Biophys. Acta, **1818**, 753-761(2012)*

Lipid rafts are membrane microdomains enriched in cholesterol, sphingolipids, and glycolipids that have been implicated in many biological processes. Since cholesterol is known to play a key role in the entry of some other viruses, we investigated the role of cholesterol and lipid rafts in the host cell plasma membrane in Newcastle Disease Virus (NDV) entry. We used methyl- β -cyclodextrin (M β CD) to deplete cellular cholesterol and disrupt lipid rafts. Our results show that the removal of cellular cholesterol partially reduces viral binding, fusion and infectivity. M β CD had no effect on the expression of sialic acid containing molecule expression, the NDV receptors in the target cell. All the above-described effects were reversed by restoring cholesterol levels in the target cell membrane. The HN viral attachment protein partially localized to detergent-resistant membrane microdomains (DRMs) at 4° C and then shifted to detergent-soluble fractions at 37° C. These results indicate that cellular cholesterol may be required for optimal cell entry in NDV infection cycle.

3.1745 S-allyl cysteine in combination with clotrimazole downregulates Fas induced apoptotic events in erythrocytes of mice exposed to lead
Mandal, S., Mukherjee, S., Chowdhury, K.D., Sarkar, A., Basu, K., Paul, S., Karmakar, D., Chatterjee, M., Biswas, T., Sadhukhan, G.C. and Sen, G.
*Biochim. Biophys. Acta, **1820**, 9-23 (2012)*

Background
Chronic lead (Pb^{2+}) exposure leads to the reduced lifespan of erythrocytes. Oxidative stress and K^+ loss accelerate Fas translocation into lipid raft microdomains inducing Fas mediated death signaling in these erythrocytes. Pathophysiological-based therapeutic strategies to combat against erythrocyte death were evaluated using garlic-derived organosulfur compounds like diallyl disulfide (DADS), S allyl cysteine (SAC) and imidazole based Gardos channel inhibitor clotrimazole (CLT).
Methods

Morphological alterations in erythrocytes were evaluated using scanning electron microscopy. Events associated with erythrocyte death were evaluated using radio labeled probes, flow cytometry and activity gel assay. Mass spectrometry was used for detection of GSH‑4-hydroxy-*trans*-2-nonenal (HNE) adducts. Fas redistribution into the lipid rafts was studied using immunoblotting technique and confocal microscopy.
Results
Combination of SAC and CLT was better than DADS and CLT combination and monotherapy with these agents in prolonging the survival of erythrocytes during chronic Pb^{2+} exposure. Combination therapy with SAC and CLT prevented redistribution of Fas into the lipid rafts of the plasma membrane and downregulated Fas-dependent death events in erythrocytes of mice exposed to Pb^{2+}.
Conclusion and general significance
Ceramide generation was a critical component of Fas receptor-induced apoptosis, since inhibition of acid sphingomyelinase (aSMase) interfered with Fas-induced apoptosis during Pb^{2+} exposure. Combination therapy with SAC and CLT downregulated apoptotic events in erythrocytes by antagonizing oxidative stress and Gardos channel that led to suppression of ceramide-initiated Fas aggregation in lipid rafts. Hence, combination therapy with SAC and CLT may be a potential therapeutic option for enhancing the lifespan of erythrocytes during Pb^{2+} toxicity.

3.1746 Cell surface ceramide controls translocation of transferrin receptor to clathrin-coated pits
Shakor, A.B.A., Atia, M.M., Kwiatkowska, K. and Sobota, A.
*Cellular Signalling, **24**, 677-684 (2012)*

Transferrin receptor mediates internalization of transferrin with bound ferric ions through the clathrin-dependent pathway. We found that binding of transferrin to the receptor induced rapid generation of cell surface ceramide which correlated with activation of acid, but not neutral, sphingomyelinase. At the onset of transferrin internalization both ceramide level and acid sphingomyelinase activity returned to their basic levels. Down-regulation of acid sphingomyelinase in cells with imipramine or silencing of the enzyme expression with siRNA stimulated transferrin internalization and inhibited its recycling. In these conditions colocalization of transferrin with clathrin was markedly reduced. Simultaneously, K^+ depletion of cells which interfered with the assembly of clathrin-coated pits inhibited the uptake of transferrin much less efficiently than it did in control conditions. The down-regulation of acid sphingomyelinase activity led to the translocation of transferrin receptor to the raft fraction of the plasma membrane upon transferrin binding. The data suggest that lack of cell surface ceramide, generated in physiological conditions by acid sphingomyelinase during transferrin binding, enables internalization of transferrin/transferrin receptor complex by clathrin-independent pathway.

3.1747 Proteostasis of tau. Tau overexpression results in its secretion via membrane vesicles
Simon, D., Garcia-garcia, E., Royo, F., Falcon-Perez, J.M. and Avila, J.
*FEBS Lett., **586**, 47-54 (2012)*

Increasing amounts of tau protein were expressed in non-neuronal cells. When intracellular amounts reached a threshold level, tau protein was released to the extracellular culture medium in association with membrane vesicles. Hence, we propose that tau might be secreted through membrane vesicles as a cellular mechanism to eliminate the excess of tau protein, thereby avoiding its toxicity.

3.1748 Perinuclear Localization of Internalized Outer Membrane Vesicles Carrying Active Cytolethal Distending Toxin from Aggregatibacter actinomycetemcomitans
Rompikuntal, P.K., Thay, B., Khan, M.K., alanko, J., Penttinen, A-M., Asikainen, S.., Wai, S.N. and Oscarsson, J.
*Infect. Immun., **80(1)**, 31-42 (2012)*

Aggregatibacter actinomycetemcomitans is implicated in aggressive forms of periodontitis. Similarly to several other Gram-negative species, this organism produces and excretes a cytolethal distending toxin (CDT), a genotoxin associated with cell distention, G_2 cell cycle arrest, and/or apoptosis in many mammalian cell types. In this study, we have identified A. actinomycetemcomitans outer membrane vesicles (OMVs) as a vehicle for simultaneous delivery of multiple proteins, including CDT, into human cells. The OMV proteins were internalized in both HeLa cells and human gingival fibroblasts (HGF) via a mechanism of OMV fusion with lipid rafts in the plasma membrane. The active toxin unit, CdtB, was localized inside the nucleus of the intoxicated cells, whereas OmpA and proteins detected using an antibody specific to whole A. actinomycetemcomitans serotype a cells had a perinuclear distribution. In

accordance with a tight association of CdtB with OMVs, vesicles isolated from A. actinomycetemcomitans strain D7SS (serotype a), in contrast to OMVs from a D7SS *cdtABC* mutant, induced a cytolethal distending effect on HeLa and HGF cells, indicating that OMV-associated CDT was biologically active. Association of CDT with OMVs was also observed in A. actinomycetemcomitans isolates belonging to serotypes b and c, indicating that OMV-mediated release of CDT may be conserved in A. actinomycetemcomitans. Although the role of A. actinomycetemcomitans OMVs in periodontal disease has not yet been elucidated, our present data suggest that OMVs could deliver biologically active CDT and additional virulence factors into susceptible cells of the periodontium.

3.1749 The Enolase of Borrelia burgdorferi Is a Plasminogen Receptor Released in Outer Membrane Vesicles

Toledo, A., Coleman, J.L., Kuhlow, C.J., Crowley, J.T. and Benach, J.L.
Infect Immun., ***80(1)***, *359-368 (2012)*

The agent of Lyme disease, Borrelia burgdorferi, has a number of outer membrane proteins that are differentially regulated during its life cycle. In addition to their physiological functions in the organism, these proteins also likely serve different functions in invasiveness and immune evasion. In borreliae, as well as in other bacteria, a number of membrane proteins have been implicated in binding plasminogen. The activation and transformation of plasminogen into its proteolytically active form, plasmin, enhances the ability of the bacteria to disseminate in the host. Outer membrane vesicles of B. burgdorferi contain enolase, a glycolytic-cycle enzyme that catalyzes 2-phosphoglycerate to form phosphoenolpyruvate, which is also a known plasminogen receptor in Gram-positive bacteria. The enolase was cloned, expressed, purified, and used to generate rabbit antienolase serum. The enolase binds plasminogen in a lysine-dependent manner but not through ionic interactions. Although it is present in the outer membrane, microscopy and proteinase K treatment showed that enolase does not appear to be exposed on the surface. However, enolase in the outer membrane vesicles is accessible to proteolytic degradation by proteinase K. Samples from experimentally and tick-infected mice and rabbits as well as from Lyme disease patients exhibit recognition of enolase in serologic assays. Thus, this immunogenic plasminogen receptor released in outer membrane vesicles could be responsible for external proteolysis in the pericellular environment and have roles in nutrition and in enhancing dissemination.

3.1750 Chromogranins A and B are key proteins in amine accumulation, but the catecholamine secretory pathway is conserved without them

Diaz-Vera, J., Camacho, M., Machado, J.D., Dominguez, N., Montesinos, M.S., Hernandez-Fernaud, J.R., Lujan, R. and Borges, R.
FASEB J., ***26(1)***, *430-438 (2012)*

Chromogranins are the main soluble proteins in the large dense core secretory vesicles (LDCVs) found in aminergic neurons and chromaffin cells. We recently demonstrated that chromogranins A and B each regulate the concentration of adrenaline in chromaffin granules and its exocytosis. Here we have further studied the role played by these proteins by generating mice lacking both chromogranins. Surprisingly, these animals are both viable and fertile. Although chromogranins are thought to be essential for their biogenesis, LDCVs were evident in these mice. These vesicles do have a somewhat atypical appearance and larger size. Despite their increased size, single-cell amperometry recordings from chromaffin cells showed that the amine content in these vesicles is reduced by half. These data demonstrate that although chromogranins regulate the amine concentration in LDCVs, they are not completely essential, and other proteins unrelated to neurosecretion, such as fibrinogen, might compensate for their loss to ensure that vesicles are generated and the secretory pathway conserved.—Díaz-Vera, J., Camacho, M., Machado, J. D., Domínguez, N., Montesinos, M. S., Hernández-Fernaud, J. R., Luján, R., Borges, R. Chromogranins A and B are key proteins in amine accumulation, but the catecholamine secretory pathway is conserved without them.

3.1751 Bromovirus RNA Replication Compartment Formation Requires Concerted Action of 1a's Self-Interacting RNA Capping and Helicase Domains

Diaz, A., Gallei, A. and Ahlquist, P.
J. Virol., ***86(2)***, *821-834 (2012)*

All positive-strand RNA viruses replicate their genomes in association with rearranged intracellular membranes such as single- or double-membrane vesicles. Brome mosaic virus (BMV) RNA synthesis

occurs in vesicular endoplasmic reticulum (ER) membrane invaginations, each induced by many copies of viral replication protein 1a, which has N-terminal RNA capping and C-terminal helicase domains. Although the capping domain is responsible for 1a membrane association and ER targeting, neither this domain nor the helicase domain was sufficient to induce replication vesicle formation. Moreover, despite their potential for mutual interaction, the capping and helicase domains showed no complementation when coexpressed in *trans*. Cross-linking showed that the capping and helicase domains each form trimers and larger multimers *in vivo*, and the capping domain formed extended, stacked, hexagonal lattices *in vivo*. Furthermore, coexpressing the capping domain blocked the ability of full-length 1a to form replication vesicles and replicate RNA and recruited full-length 1a into mixed hexagonal lattices with the capping domain. Thus, BMV replication vesicle formation and RNA replication depend on the direct linkage and concerted action of 1a's self-interacting capping and helicase domains. In particular, the capping domain's strong dominant-negative effects showed that the ability of full-length 1a to form replication vesicles was highly sensitive to disruption by non-productively titrating lattice-forming self-interactions of the capping domain. These and other findings shed light on the roles and interactions of 1a domains in replication compartment formation and support prior results suggesting that 1a induces replication vesicles by forming a capsid-like interior shell.

3.1752 Streptococcus suis Capsular Polysaccharide Inhibits Phagocytosis through Destabilization of Lipid Microdomains and Prevents Lactosylceramide-Dependent Recognition

Houde, M., Gottschalk, M., Gagnon, F., Van Calsteren, M-R. and Segura, M.
*Infect. Immun.,**80(2)**, 506-517 (2012)*

Streptococcus suis type 2 is a major swine pathogen and a zoonotic agent, causing meningitis in both swine and humans. S. suis infects the host through the respiratory route, reaches the bloodstream, and persists until breaching into the central nervous system. The capsular polysaccharide (CPS) of S. suis type 2 is considered a key virulence factor of the bacteria. Though CPS allows S. suis to adhere to the membrane of cells of the immune system, it provides protection against phagocytosis. In fact, nonencapsulated mutants are easily internalized and killed by macrophages and dendritic cells. The objective of this work was to study the molecular mechanisms by which the CPS of S. suis prevents phagocytosis. By using latex beads covalently linked with purified CPS, it was shown that CPS itself was sufficient to inhibit entry of both latex beads and bystander fluorescent beads into macrophages. Upon contact with macrophages, encapsulated S. suis was shown to destabilize lipid microdomains at the cell surface, to block nitric oxide (NO) production during infection, and to prevent lactosylceramide accumulation at the phagocytic cup during infection. In contrast, the nonencapsulated mutant was easily internalized via lipid rafts, in a filipin-sensitive manner, leading to lactosylceramide recruitment and strong NO production. This is the first report to identify a role for CPS in lipid microdomain stability and to recognize an interaction between S. suis and lactosylceramide in phagocytes.

3.1753 HIV-1 Vpu's lipid raft association is dispensable for counteraction of the particle release restriction imposed by CD317/Tetherin

Joëlle, V.F., Tibroni, N., Keppler, O.T. and Fackler, O.T.
*Virology, **424**, 33-44 (2012)*

HIV-1 Vpu antagonizes the block to particle release mediated by CD317 (BST-2/HM1.24/Tetherin) via incompletely understood mechanisms. Vpu and CD317 partially reside in cholesterol-rich lipid rafts where HIV-1 budding preferentially occurs. Here we find that lipid raft association of ectopically expressed or endogenous CD317 was unaltered upon co-expression with Vpu or following HIV-1 infection. Similarly, Vpu's lipid raft association remained unchanged upon expression of CD317. We identify amino acids V25 and Y29 of Vpu as crucial for microdomain partitioning and single substitution of these amino acids resulted in Vpu variants with markedly reduced or undetectable lipid raft association. These mutations did not affect Vpu's subcellular distribution and binding capacity to CD317, nor its ability to downmodulate cell surface CD317 and promote HIV-1 release from CD317-positive cells. We conclude that (i) lipid raft incorporation is dispensable for Vpu-mediated CD317 antagonism and (ii) Vpu does not antagonize CD317 by extraction from lipid rafts.

3.1754 Immune cells and hepatocytes express glycosylphosphatidylinositol-anchored ceruloplasmin at their cell surface

Marques, L., Auriac, A., Willemetz, A., Banha, J., Silva, B., Canonne-Hergaux, F. and Costa, L.
*Blood Cells, Molecules, and Diseases, **48**, 110-120 (2012)*

Background
Ceruloplasmin is a positive acute-phase protein with both anti- and pro-oxidant activities, thus having still unclear physiological functions in inflammatory processes. Importantly, ceruloplasmin has been implicated in iron metabolism due to its ferroxidase activity, assisting ferroportin on cellular iron efflux. Ceruloplasmin can be expressed as a secreted or as a membrane glycosylphosphatidylinositol-anchored protein (GPI-ceruloplasmin), this latter one being reported as expressed mostly in the brain.
Design and methods
We studied the expression of both ceruloplasmin isoforms in human peripheral blood lymphocytes, monocytes, mouse macrophages and human hepatocarcinoma cell line HepG2, using immunofluorescence and immunoblotting techniques. Co-localization of ceruloplasmin and ferroportin was also investigated by immunofluorescence in mouse macrophages.
Results
Ceruloplasmin was detected by immunoblotting and immunofluorescence in membrane and cytosol of all cell types. The cell surface ceruloplasmin was identified as the GPI-isoform and localized in lipid rafts from monocytes, macrophages and HepG2 cells. In macrophages, increased expression levels and co-localization of ferroportin and GPI-ceruloplasmin in cell surface lipid rafts were observed after iron treatment. Such iron upregulation of ceruloplasmin was not observed in HepG2.
Conclusions
Our results revealed an unexpected ubiquitous expression of the GPI-ceruloplasmin isoform in immune and hepatic cells. Different patterns of regulation of ceruloplasmin in these cells may reflect distinct physiologic functions of this oxidase. In macrophages, GPI-ceruloplasmin and ferroportin likely interact in lipid rafts to export iron from cells. Precise knowledge about ceruloplasmin isoforms expression and function in various cell types will help to clarify the role of ceruloplasmin in many diseases related to iron metabolism, inflammation and oxidative biology.

3.1755 Baculovirus GP64-Mediated Entry into Mammalian Cells
Kataoka, C., Kaname, Y., Taguwa, S., Abe, T., Fukuhara, T., Tani, H., Moriishi, K and Matsuura, Y.
J. Virol., ***86(5)***, *2610-2620 (2012)*

The baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) serves as an efficient viral vector, not only for abundant gene expression in insect cells, but also for gene delivery into mammalian cells. Lentivirus vectors pseudotyped with the baculovirus envelope glycoprotein GP64 have been shown to acquire more potent gene transduction than those with vesicular stomatitis virus (VSV) envelope glycoprotein G. However, there are conflicting hypotheses about the molecular mechanisms of the entry of AcMNPV. Moreover, the mechanisms of the entry of pseudotyped viruses bearing GP64 into mammalian cells are not well characterized. Determination of the entry mechanisms of AcMNPV and the pseudotyped viruses bearing GP64 is important for future development of viral vectors that can deliver genes into mammalian cells with greater efficiency and specificity. In this study, we generated three pseudotyped VSVs, NPVpv, VSVpv, and MLVpv, bearing envelope proteins of AcMNPV, VSV, and murine leukemia virus, respectively. Depletion of membrane cholesterol by treatment with methyl-β-cyclodextrin, which removes cholesterol from cellular membranes, inhibited GP64-mediated internalization in a dose-dependent manner but did not inhibit attachment to the cell surface. Treatment of cells with inhibitors or the expression of dominant-negative mutants for dynamin- and clathrin-mediated endocytosis abrogated the internalization of AcMNPV and NPVpv into mammalian cells, whereas inhibition of caveolin-mediated endocytosis did not. Furthermore, inhibition of macropinocytosis reduced GP64-mediated internalization. These results suggest that cholesterol in the plasma membrane, dynamin- and clathrin-dependent endocytosis, and macropinocytosis play crucial roles in the entry of viruses bearing baculovirus GP64 into mammalian cells.

3.1756 Role of protein kinase C in phospholemman mediated regulation of α2β1 isozyme of Na+/K+-ATPase in caveolae of pulmonary artery smooth muscle cells
Dey, K., Roy, S., Ghosh, B. and Chakraborti, S.
Biochemie, ***94***, *991-1000 (2012)*

We have recently reported that $\alpha_2\beta_1$ and $\alpha_1\beta_1$ isozymes of Na^+/K^+-ATPase (NKA) are localized in the caveolae whereas only the $\alpha_1\beta_1$ isozyme of NKA is localized in the non-caveolae fraction of pulmonary artery smooth muscle cell membrane. It is well known that different isoforms of NKA are regulated differentially by PKA and PKC, but the mechanism is not known in the caveolae of pulmonary artery smooth muscle cells. Herein, we examined whether this regulation occurs through phospholemman (PLM) in the caveolae. Our results suggest that PKC mediated phosphorylation of PLM occurs only when it is

associated with the α_2 isoform of NKA, whereas phosphorylation of PLM by PKA occurs when it is associated with the α_1 isoform of NKA. To investigate the mechanism of regulation of α_2 isoform of NKA by PKC-mediated phosphorylation of PLM, we have purified PLM from the caveolae and reconstituted into the liposomes. Our result revealed that (i) in the reconstituted liposomes phosphorylated PLM (PKC mediated) stimulate NKA activity, which appears to be due to an increase in the turnover number of the enzyme; (ii) phosphorylated PLM did not change the affinity of the pump for Na^+; and (iii) even after phosphorylation by PKC, PLM still remains associated with the α_2 isoform of NKA.

3.1757 Palmitoylated TMX and calnexin target to the mitochondria-associated membrane
Lynes, E.M., Bui, M., Yap, M.C., Benson, M.D., Schneider, B., Ellgaard, L., Berthiaume, L.C. and Simmen, T.
EMBO J., ***31(2)***, *457-470 (2012)*

The mitochondria-associated membrane (MAM) is a domain of the endoplasmic reticulum (ER) that mediates the exchange of ions, lipids and metabolites between the ER and mitochondria. ER chaperones and oxidoreductases are critical components of the MAM. However, the localization motifs and mechanisms for most MAM proteins have remained elusive. Using two highly related ER oxidoreductases as a model system, we now show that palmitoylation enriches ER-localized proteins on the MAM. We demonstrate that palmitoylation of cysteine residue(s) adjacent to the membrane-spanning domain promotes MAM enrichment of the transmembrane thioredoxin family protein TMX. In addition to TMX, our results also show that calnexin shuttles between the rough ER and the MAM depending on its palmitoylation status. Mutation of the TMX and calnexin palmitoylation sites and chemical interference with palmitoylation disrupt their MAM enrichment. Since ER-localized heme oxygenase-1, but not cytosolic GRP75 require palmitoylation to reside on the MAM, our findings identify palmitoylation as key for MAM enrichment of ER membrane proteins.

3.1758 Assurance of mitochondrial integrity and mammalian longevity by the p62–Keap1–Nrf2–Nqo1 cascade
Kwon, J., Han, E., Bui, C-B., Shin, W., Lee, J., Lee, S., Choi, Y-B., Lee, A-H., Lee, K-H., Park, C., Obin, M.S., Park, S.K., Seo, Y.J., Taeg, G., Lee, H-W. and Shin, J.
EMBO Reports, ***13(2)***, *150-156 (2012)*

Sqstm1/p62 functions in the non-canonical activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2). However, its physiological relevance is not certain. Here, we show that *p62* $^{-/-}$ mice exhibited an accelerated presentation of ageing phenotypes, and tissues from these mice created a pro-oxidative environment owing to compromised mitochondrial electron transport. Accordingly, mitochondrial function rapidly declined with age in *p62* $^{-/-}$ mice. In addition, p62 enhanced basal Nrf2 activity, conferring a higher steady-state expression of NAD(P)H dehydrogenase, quinone 1 (Nqo1) to maintain mitochondrial membrane potential and, thereby, restrict excess oxidant generation. Together, the p62–Nrf2–Nqo1 cascade functions to assure mammalian longevity by stabilizing mitochondrial integrity.

3.1759 A conserved membrane-binding domain targets proteins to organelle contact sites
Toulmay, A. and Printz, W.A.
J. Cell Sci., ***125(1)***, *49-58 (2012)*

Membrane contact sites (MCSs), where the membranes of two organelles are closely apposed, are regions where small molecules such as lipids or calcium are exchanged between organelles. We have identified a conserved membrane-binding domain found exclusively in proteins at MCSs in *Saccharomyces cerevisiae*. The synaptotagmin-like-mitochondrial-lipid binding protein (SMP) domain is conserved across species. We show that all seven proteins that contain this domain in yeast localize to one of three MCSs. Human proteins with SMP domains also localize to MCSs when expressed in yeast. The SMP domain binds membranes and is necessary for protein targeting to MCSs. Proteins containing this domain could be involved in lipid metabolism. This is the first protein domain found exclusively in proteins at MCSs.

3.1760 Quantitative analysis of the lipidomes of the influenza virus envelope and MDCK cell apical membrane
Gerl, M.J., Sampaio, J.L., Urban, S., Kalvodova, L., Verbavatz, J-M., Binnington, B., Lindemann, D., Lingwood, C.A., Shevchenko, A., Schroder, C. and Simons, K.
J. Cell Biol., ***196(2)***, *213-221 (2012)*

The influenza virus (IFV) acquires its envelope by budding from host cell plasma membranes. Using quantitative shotgun mass spectrometry, we determined the lipidomes of the host Madin–Darby canine kidney cell, its apical membrane, and the IFV budding from it. We found the apical membrane to be enriched in sphingolipids (SPs) and cholesterol, whereas glycerophospholipids were reduced, and storage lipids were depleted compared with the whole-cell membranes. The virus membrane exhibited a further enrichment of SPs and cholesterol compared with the donor membrane at the expense of phosphatidylcholines. Our data are consistent with and extend existing models of membrane raft-based biogenesis of the apical membrane and IFV envelope.

3.1761 **Building Excitable Membranes: Lipid Rafts and Multiple Controls on Trafficking of Electrogenic Molecules**
Pristera, A. and Okuse, K.
*The Neuroscientist, **18(1)**, 70-81 (2012)*

Multiple plasma membrane proteins such as ion transporters and ion channels are involved in electrogenesis by setting resting membrane potentials and triggering/propagating action potentials. Recent findings strongly suggest that some of these membrane proteins are selectively transported into membrane microdomains termed *lipid rafts*. There appear to be multiple mechanisms for the specific protein translocation to lipid rafts, and many of these proteins exhibit distinct properties when inserted into the raft microdomains. Here the authors review the plasma membrane ion channels specifically localized at membrane lipid rafts in neurons. The mechanisms to selectively translocate these molecules to the lipid rafts and the consequences of the trafficking are also discussed.

3.1762 **Tau deficiency induces parkinsonism with dementia by impairing APP-mediated iron export**
Lee, P. et al
*Nature Med., **18(2)**, 291-296 (2012)*

The microtubule-associated protein tau has risk alleles for both Alzheimer's disease and Parkinson's disease and mutations that cause brain degenerative diseases termed tauopathies[1, 2, 3, 4]. Aggregated tau forms neurofibrillary tangles in these pathologies[3, 5], but little is certain about the function of tau or its mode of involvement in pathogenesis. Neuronal iron accumulation has been observed pathologically in the cortex in Alzheimer's disease[6, 7], the substantia nigra (SN) in Parkinson's disease[8, 9, 10, 11] and various brain regions in the tauopathies[11, 12]. Here we report that tau-knockout mice develop age-dependent brain atrophy, iron accumulation and SN neuronal loss, with concomitant cognitive deficits and parkinsonism. These changes are prevented by oral treatment with a moderate iron chelator, clioquinol. Amyloid precursor protein (APP) ferroxidase activity couples with surface ferroportin to export iron, but its activity is inhibited in Alzheimer's disease, thereby causing neuronal iron accumulation[7]. In primary neuronal culture, we found loss of tau also causes iron retention, by decreasing surface trafficking of APP. Soluble tau levels fall in affected brain regions in Alzheimer's disease and tauopathies[13, 14, 15], and we found a similar decrease of soluble tau in the SN in both Parkinson's disease and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model. These data suggest that the loss of soluble tau could contribute to toxic neuronal iron accumulation in Alzheimer's disease, Parkinson's disease and tauopathies, and that it can be rescued pharmacologically.

3.1763 **Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles**
Yao, J., Kwon, S.E., Gaffaney, J.D., Dunning, F.M. and Chapman, E.R.
*Nature Neurosci., **15(2)**, 243-250 (2012)*

Synaptotagmin I (syt1) is required for normal rates of synaptic vesicle endo- and exocytosis. However, whether the kinetic defects observed during endocytosis in *Syt1* knockout neurons are secondary to defective exocytosis or whether syt1 directly regulates the rate of vesicle retrieval remains unknown. To address this question, we sought to dissociate these two activities. We uncoupled the function of syt1 in exo- and endocytosis in mouse neurons either by re-targeting the protein or via mutagenesis of its tandem C2 domains. The effect of these manipulations on exo- and endocytosis were analyzed using electrophysiology, in conjunction with optical imaging of the vesicle cycle. Our results indicate that syt1 is directly involved in endocytosis. Notably, either of the C2 domains of syt1, C2A or C2B, was able to function as a Ca^{2+} sensor for endocytosis. Thus, syt1 functions as a dual Ca^{2+} sensor for both endo- and exocytosis, potentially coupling these two components of the vesicle cycle.

3.1764 Cullin-3 regulates late endosome maturation

Huotari, J., Meyer-Schaller, N., Hubner, M., Stauffer, S., Katheder, N., Horvath, P., Mancini, r., Heleniius, A. and Peter, M.

*PNAS, **19(3)**, 823-828 (2012)*

Cullin-3 (Cul3) functions as a scaffolding protein in the Bric-a-brac, Tramtrack, Broad-complex (BTB)–Cul3–Rbx1 ubiquitin E3 ligase complex. Here, we report a previously undescribed role for Cul3 complexes in late endosome (LE) maturation. RNAi-mediated depletion of Cul3 results in a trafficking defect of two cargoes of the endolysosomal pathway, influenza A virus (IAV) and epidermal growth factor receptor (EGFR). IAV is able to reach an acidic endosomal compartment, coinciding with LE/lysosome (LY) markers. However, it remains trapped or the capsid is unable to uncoat after penetration into the cytosol. Similarly, activation and subsequent ubiquitination of EGFR appear normal, whereas downstream EGFR degradation is delayed and its ligand EGF accumulates in LE/LYs. Indeed, Cul3-depleted cells display severe morphological defects in LEs that could account for these trafficking defects; they accumulate acidic LE/LYs, and some cells become highly vacuolated, with enlarged Rab7-positive endosomes. Together, these results suggest a crucial role of Cul3 in regulating late steps in the endolysosomal trafficking pathway.

3.1765 Tempol modulates changes in xenobiotic permeability and occludin oligomeric assemblies at the blood-brain barrier during inflammatory pain

Lockhead, J.J., McCaffrey, G., Sanchez-Covarrubias, L., Finch, J.D., DeMarco, K.M., Quigley, C.E., Davis, T.P. and Ronaldson, P.T.

*Am. J. Physiol. Heart Circ. Physiol., **302(3)**, H582-H593 (2012)*

Our laboratory has shown that λ-carrageenan-induced peripheral inflammatory pain (CIP) can alter tight junction (TJ) protein expression and/or assembly leading to changes in blood-brain barrier xenobiotic permeability. However, the role of reactive oxygen species (ROS) and subsequent oxidative stress during CIP is unknown. ROS (i.e., superoxide) are known to cause cellular damage in response to pain/inflammation. Therefore, we examined oxidative stress-associated effects at the blood-brain barrier (BBB) in CIP rats. During CIP, increased staining of nitrosylated proteins was detected in hind paw tissue and enhanced presence of protein adducts containing 3-nitrotyrosine occurred at two molecular weights (i.e., 85 and 44 kDa) in brain microvessels. Tempol, a pharmacological ROS scavenger, attenuated formation of 3-nitrotyrosine-containing proteins in both the hind paw and in brain microvessels when administered 10 min before footpad injection of λ-carrageenan. Similarly, CIP increased 4-hydroxynoneal staining in brain microvessels and this effect was reduced by tempol. Brain permeability to [^{14}C]sucrose and [^{3}H]codeine was increased, and oligomeric assemblies of occludin, a critical TJ protein, were altered after 3 h CIP. Tempol attenuated both [^{14}C]sucrose and [^{3}H]codeine brain uptake as well as protected occludin oligomers from disruption in CIP animals, suggesting that ROS production/oxidative stress is involved in modulating BBB functional integrity during pain/inflammation. Interestingly, tempol administration reduced codeine analgesia in CIP animals, indicating that oxidative stress during pain/inflammation may affect opioid delivery to the brain and subsequent efficacy. Taken together, our data show for the first time that ROS pharmacological scavenging is a viable approach for maintaining BBB integrity and controlling central nervous system drug delivery during acute inflammatory pain.

3.1766 Proteomic Analysis of Microvesicles Derived from Human Mesenchymal Stem Cells

Kim, H-S., Choi, D-Y., Yun, S.J., Choi, S-M., kang, J.W., Jung, J.W., Hwang, D., Kim, K.P. and Kim, D-W.,

*J. Proteome Res., **11(2)**, 839-849 (2012)*

Mesenchymal stem cells (MSCs) have emerged as a promising means for treating degenerative or incurable diseases. Recent studies have shown that microvesicles (MVs) from MSCs (MSC-MVs) contribute to recovery of damaged tissues in animal disease models. Here, we profiled the MSC-MV proteome to investigate their therapeutic effects. LC–MS/MS analysis of MSC-MVs identified 730 MV proteins. The MSC-MV proteome included five positive and two variable known markers of MSCs, but no negative marker, as well as 43 surface receptors and signaling molecules controlling self-renewal and differentiation of MSCs. Functional enrichment analysis showed that cellular processes represented by the MSC-MV proteins include cell proliferation, adhesion, migration, and morphogenesis. Integration of MSC's self-renewal and differentiation-related genes and the proteome of MSC-conditioned media (MSC-

CM) with the MSC-MV proteome revealed potential MV protein candidates that can be associated with the therapeutic effects of MSC-MVs: (1) surface receptors (PDGFRB, EGFR, and PLAUR); (2) signaling molecules (RRAS/NRAS, MAPK1, GNA13/GNG12, CDC42, and VAV2); (3) cell adhesion (FN1, EZR, IQGAP1, CD47, integrins, and LGALS1/LGALS3); and (4) MSC-associated antigens (CD9, CD63, CD81, CD109, CD151, CD248, and CD276). Therefore, the MSC-MV proteome provides a comprehensive basis for understanding the potential of MSC-MVs to affect tissue repair and regeneration.

3.1767 **Angiotensin II Induces Epithelial-to-Mesenchymal Transition in Renal Epithelial Cells through Reactive Oxygen Species/Src/Caveolin-Mediated Activation of an Epidermal Growth Factor Receptor–Extracellular Signal-Regulated Kinase Signaling Pathway**
Chen, J., Chen, J-K. and Harris, R.C.
Mol. Cell. Biol., ***32(5)***, *981-991 (2012)*

Chronic activation of the renin-angiotensin system plays a deleterious role in progressive kidney damage, and the renal proximal tubule is known to play an important role in tubulointerstitial fibrosis; however, the underlying molecular mechanism is unclear. Here we report that in the proximal tubule-like LLCPKcl4 cells expressing angiotensin II (Ang II) type 1 receptor, Ang II induced changes in cell morphology and expression of epithelial-to-mesenchymal transition (EMT) markers, which were inhibited by the miotogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK)-activating kinase (MEK) inhibitor PD98059 or the Src kinase inhibitor PP2. Ang II-stimulated phosphorylation of caveolin-1 (Cav) at Y14 and epidermal growth factor receptor (EGFR) at Y845 and induced association of these phosphoproteins in caveolin-enriched lipid rafts, thereby leading to prolonged EGFR-ERK signaling that was inhibited by Nox4 small interfering RNA (siRNA) and Src siRNA. Two different antioxidants not only inhibited phosphorylation of Src at Y416 but also blocked the EGFR-ERK signaling. Moreover, erlotinib (the EGFR tyrosine kinase inhibitor), EGFR siRNA, and Cav siRNA all inhibited both prolonged EGFR-ERK signaling and phenotypic changes induced by Ang II. Thus, this report provides the first evidence that reactive oxygen species (ROS)/Src-dependent activation of persistent Cav-EGFR-ERK signaling mediates renal tubular cell dedifferentiation and identifies a novel molecular mechanism that may be involved in progressive renal injury caused by chronic exposure to Ang II.

3.1768 **Measuring and evaluating the role of ATP-sensitive K+ channels in cardiac muscle**
Kefaloyianni, E., Bao, L., Rindler, M.J., Hong, M., Patel, T., Taskin, E. and Coetzee, W.A.
J. Mol. Cell. Cardiol., ***52***, *596-607 (2012)*

Since ion channels move electrical charge during their activity, they have traditionally been studied using electrophysiological approaches. This was sometimes combined with mathematical models, for example with the description of the ionic mechanisms underlying the initiation and propagation of action potentials in the squid giant axon by Hodgkin and Huxley. The methods for studying ion channels also have strong roots in protein chemistry (limited proteolysis, the use of antibodies, etc.). The advent of the molecular cloning and the identification of genes coding for specific ion channel subunits in the late 1980s introduced a multitude of new techniques with which to study ion channels and the field has been rapidly expanding ever since (e.g. antibody development against specific peptide sequences, mutagenesis, the use of gene targeting in animal models, determination of their protein structures) and new methods are still in development. This review focuses on techniques commonly employed to examine ion channel function in an electrophysiological laboratory. The focus is on the K_{ATP} channel, but many of the techniques described are also used to study other ion channels.

3.1769 **Endoplasmic Reticulum Stress Is Important for the Manifestations of α-Synucleinopathy In Vivo**
Colla, E., Coune, P., Liu, Y., Pletnikova, O., Troncoso, J.C., Iwatsubo, T., Schneider, B.L. and Lee, M.K.
J. Neurosci., ***32(10)***, *3306-3320 (2012)*

Accumulation of misfolded α-synuclein (αS) is mechanistically linked to neurodegeneration in Parkinson's disease (PD) and other α-synucleinopathies. However, how αS causes neurodegeneration is unresolved. Because cellular accumulation of misfolded proteins can lead to endoplasmic reticulum stress/unfolded protein response (ERS/UPR), chronic ERS could contribute to neurodegeneration in α-synucleinopathy. Using the A53T mutant human αS transgenic (*A53TαS* Tg) mouse model of α-synucleinopathy, we show that disease onset in the αS Tg model is coincident with induction of ER chaperones in neurons exhibiting αS pathology. However, the neuronal ER chaperone induction was not accompanied by the activation of phospho-eIF2α, indicating that α-synucleinopathy is associated with abnormal UPR that could promote cell death. Induction of ERS/UPR was associated with increased levels of ER/microsomal (ER/M)

associated αS monomers and aggregates. Significantly, human PD cases also exhibit higher relative levels of ER/M αS than the control cases. Moreover, αS interacts with ER chaperones and overexpression of αS sensitizes neuronal cells to ERS-induced toxicity, suggesting that αS may have direct impact on ER function. This view is supported by the presence of ERS-activated caspase-12 and the accumulation of ER-associated polyubiquitin. More important, treatment with Salubrinal, an anti-ERS compound, significantly attenuates disease manifestations in both the *A53TαS* Tg mouse model and the adeno-associated virus-transduced rat model of *A53TαS*-dependent dopaminergic neurodegeneration. Our data indicate that the accumulation αS within ER leads to chronic ER stress conditions that contribute to neurodegeneration in α-synucleinopathies. Attenuating chronic ERS could be an effective therapy for PD and other α-synucleinopathies.

3.1770 FBL2 Regulates Amyloid Precursor Protein (APP) Metabolism by Promoting Ubiquitination-Dependent APP Degradation and Inhibition of APP Endocytosis
Watanabe, T., Hikichi, Y., Willuweit, A., Shintani, Y. and Horiguchi, T.
J. Neurosci., ***32(10)***, *3352-3365 (2012)*

The ubiquitin–proteasome pathway is a major protein degradation pathway whose dysfunction is now widely accepted as a cause of neurodegenerative diseases, including Alzheimer's disease. Here we demonstrate that the F-box and leucine rich repeat protein2 (FBL2), a component of the E3 ubiquitin ligase complex, regulates amyloid precursor protein (APP) metabolism through APP ubiquitination. FBL2 overexpression decreased the amount of secreted amyloid β (Aβ) peptides and sAPPβ, whereas FBL2 mRNA knockdown by siRNA increased these levels. FBL2 overexpression also decreased the amount of intracellular Aβ in Neuro2a cells stably expressing APP with Swedish mutation. FBL2 bound with APP specifically at its C-terminal fragment (CTF), which promoted APP/CTF ubiquitination. FBL2 overexpression also accelerated APP proteasome-dependent degradation and decreased APP protein localization in lipid rafts by inhibiting endocytosis. These effects were not observed in an F-box-deleted FBL2 mutant that does not participate in the E3 ubiquitin ligase complex. Furthermore, a reduced insoluble Aβ and Aβ plaque burden was observed in the hippocampus of 7-month-old FBL2 transgenic mice crossed with double-transgenic mice harboring APPswe and $PS1_{M146V}$ transgenes. These findings indicate that FBL2 is a novel and dual regulator of APP metabolism through FBL2-dependent ubiquitination of APP.

3.1771 Review: Novel roles of nuclear angiotensin receptors and signaling mechanisms
Gwathmey, T.T.M., Alzayadneh, E.M., Pendergrass, K.D. and Chappell, M.C.
Am. J. Physiol. Reegul. Integr. Comp. Physiol., ***302***, *R518-R530 (2012)*

The renin-angiotensin system (RAS) constitutes an important hormonal system in the physiological regulation of blood pressure. The dysregulation of the RAS is considered a major influence in the development and progression of cardiovascular disease and other pathologies. Indeed, experimental and clinical evidence indicates that blockade of this system with angiotensin-converting enzyme (ACE) inhibitors or angiotensin type 1 receptor (AT_1R) antagonists is an effective therapy to attenuate hypertension and diabetic renal injury, and to improve heart failure. Originally defined as a circulating system, multiple tissues express a complete RAS, and compelling evidence now favors an intracellular system involved in cell signaling and function. Within the kidney, intracellular expression of the three predominant ANG receptor subtypes is evident in the nuclear compartment. The ANG type 1 receptor (AT_1R) is coupled to the generation of reactive oxygen species (ROS) through the activation of phosphoinositol-3 kinase (PI3K) and PKC. In contrast, both ANG type 2 (AT_2R) and ANG-(1–7) (AT_7R) receptors stimulate nitric oxide (NO) formation, which may involve nuclear endothelial NO synthase (eNOS). Moreover, blockade of either ACE2—the enzyme that converts ANG II to ANG-(1–7)—or the AT_7 receptor exacerbates the ANG II-ROS response on renal nuclei. Finally, in a model of fetal programmed hypertension, the nuclear ROS response to ANG II is enhanced, while both AT_2 and AT_7 stimulation of NO is attenuated, suggesting that an imbalance in the intracellular RAS may contribute to the development of programming events. We conclude that a functional intracellular or nuclear RAS may have important implications in the therapeutic approaches to cardiovascular disease.

3.1772 Regulation of invadopodia formation and activity by CD147
Grass, G.D., Bratoeva, M. and Toole, B.P.
J. Cell Sci., ***125***, *777-788 (2012)*

A defining feature of malignant tumor progression is cellular penetration through the basement membrane and interstitial matrices that separate various cellular compartments. Accumulating evidence supports the

notion that invasive cells employ specialized structures termed invadopodia to breach these structural barriers. Invadopodia are actin-based, lipid-raft-enriched membrane protrusions containing membrane-type-1 matrix metalloproteinase (MT1-MMP; also known as matrix metalloproteinase 14; MMP14) and several signaling proteins. CD147 (emmprin, basigin), an immunoglobulin superfamily protein that is associated with tumor invasion and metastasis, induces the synthesis of various matrix metalloproteinases in many systems. In this study we show that upregulation of CD147 is sufficient to induce MT1-MMP expression, invasiveness and formation of invadopodia-like structures in non-transformed, non-invasive, breast epithelial cells. We also demonstrate that CD147 and MT1-MMP are in close proximity within these invadopodia-like structures and co-fractionate in membrane compartments with the properties of lipid rafts. Moreover, manipulation of CD147 levels in invasive breast carcinoma cells causes corresponding changes in MT1-MMP expression, invasiveness and invadopodia formation and activity. These findings indicate that CD147 regulates invadopodia formation and activity, probably through assembly of MT1-MMP-containing complexes within lipid-raft domains of the invadopodia.

3.1773 Mammalian Atg2 proteins are essential for autophagosome formation and important for regulation of size and distribution of lipid droplets

Velikkakath, A.K.G., Nishimura, T., Oita, E., Ishihara, N. and Mizushima, N.
Mol. Biol. Cell, 2, 896-909 (2012)

Macroautophagy is an intracellular degradation system by which cytoplasmic materials are enclosed by the autophagosome and delivered to the lysosome. Autophagosome formation is considered to take place on the endoplasmic reticulum and involves functions of autophagy-related (Atg) proteins. Here, we report the identification and characterization of mammalian Atg2 homologues Atg2A and Atg2B. Simultaneous silencing of Atg2A and Atg2B causes a block in autophagic flux and accumulation of unclosed autophagic structures containing most Atg proteins. Atg2A localizes on the autophagic membrane, as well as on the surface of lipid droplets. The Atg2A region containing amino acids 1723–1829, which shows relatively high conservation among species, is required for localization to both the autophagic membrane and lipid droplet and is also essential for autophagy. Depletion of both Atg2A and Atg2B causes clustering of enlarged lipid droplets in an autophagy-independent manner. These data suggest that mammalian Atg2 proteins function both in autophagosome formation and regulation of lipid droplet morphology and dispersion.

3.1774 Impaired CFTR-Dependent Amplification of FSH-Stimulated Estrogen Production in Cystic Fibrosis and PCOS

Chen, H., Guo, J.H., Lu, Y.C., Ding, G.L., Yu, M.K., Tsang, L.L., Fok, K.L., Liu, X.M., Zhang, X.H., Chung, Y.W., Huang, P., Huang, H. and Chan, C.
*J. Clin. Endocrinol. Metab., **97(3)**, 923-932 (2012)*

Context: Estrogens play important roles in a wide range of physiological and pathological processes, and their biosynthesis is profoundly influenced by FSH that regulates the rate-limiting enzyme aromatase-converting estrogens from androgens. Abnormal estrogen levels are often seen in diseases such as ovarian disorders in polycystic ovarian syndrome (PCOS), an endocrine disorder affecting 5–10% of women of reproductive age, and cystic fibrosis (CF), a common genetic disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR).
Objectives: We undertook the present study to investigate the mechanism underlying these ovarian disorders, which is not well understood.
Results: FSH-stimulated cAMP-responsive element binding protein phosphorylation, aromatase expression, and estradiol production are found to be enhanced by HCO_3^- and a HCO_3^- sensor, the soluble adenylyl cyclase, which could be significantly reduced by CFTR inhibition or in ovaries or granulosa cells of cftr knockout/ΔF508 mutant mice. CFTR expression is found positively correlated with aromatase expression in human granulosa cells, supporting its role in regulating estrogen production in humans. Reduced CFTR and aromatase expression is also found in PCOS rodent models and human patients.
Conclusions: CFTR regulates ovarian estrogen biosynthesis by amplifying the FSH-stimulated signal via the nuclear soluble adenylyl cyclase. The present findings suggest that defective CFTR-dependent regulation of estrogen production may underlie the ovarian disorders seen in CF and PCOS.

3.1775 Co-Regulation of Transcellular and Paracellular Leak Across Microvascular Endothelium by Dynamin and Rac

Armstrong, S.M., Khajoee, V., Wang, C., Wang, ST., Tigdi, J., Yin, J., Kuebler, W.M., Gillrie, M., Davis, S.P., Ho, M. and Lee, W.L.

Am. J. Pathol., ***180(3)***, *1308-1323 (2012)*

Increased permeability of the microvascular endothelium to fluids and proteins is the hallmark of inflammatory conditions such as sepsis. Leakage can occur between (paracellular) or through (transcytosis) endothelial cells, yet little is known about whether these pathways are linked. Understanding the regulation of microvascular permeability is essential for the identification of novel therapies to combat inflammation. We investigated whether transcytosis and paracellular leakage are co-regulated. Using molecular and pharmacologic approaches, we inhibited transcytosis of albumin in primary human microvascular endothelium and measured paracellular permeability. Blockade of transcytosis induced a rapid increase in paracellular leakage that was not explained by decreases in caveolin-1 or increases in activity of nitric oxide synthase. The effect required caveolin-1 but was observed in cells depleted of clathrin, indicating that it was not due to the general inhibition of endocytosis. Inhibiting transcytosis by dynamin blockade increased paracellular leakage concomitantly with the loss of cortical actin from the plasma membrane and the displacement of active Rac from the plasmalemma. Importantly, inhibition of paracellular leakage by sphingosine-1-phosphate, which activates Rac and induces cortical actin, caused a significant increase in transcytosis of albumin *in vitro* and in an *ex vivo* whole-lung model. In addition, dominant-negative Rac significantly diminished albumin uptake by endothelia. Our findings indicate that transcytosis and paracellular permeability are co-regulated through a signaling pathway linking dynamin, Rac, and actin.

3.1776 HIV-1 Nef mobilizes lipid rafts in macrophages through a pathway that competes with ABCA1-dependent cholesterol efflux

Cui, H.L., Grant, A., Mukhamedova, N., Pushkarsky, T., Jenelle, L., Dubrovsky, L., Gaus, K., Fitzgerald, M.L., Sviridov, D. and Bukrinsky, M.

HIV infection, through the actions of viral accessory protein Nef, impairs activity of cholesterol transporter ABCA1, inhibiting cholesterol efflux from macrophages and elevating the risk of atherosclerosis. Nef also induces lipid raft formation. In this study, we demonstrate that these activities are tightly linked and affect macrophage function and HIV replication. Nef stimulated lipid raft formation in macrophage cell line RAW 264.7, and lipid rafts were also mobilized in HIV-1-infected human monocyte-derived macrophages. Nef-mediated transfer of cholesterol to lipid rafts competed with the ABCA1-dependent pathway of cholesterol efflux, and pharmacological inhibition of ABCA1 functionality or suppression of ABCA1 expression by RNAi increased Nef-dependent delivery of cholesterol to lipid rafts. Nef reduced cell-surface accessibility of ABCA1 and induced ABCA1 catabolism via the lysosomal pathway. Despite increasing the abundance of lipid rafts, expression of Nef impaired phagocytic functions of macrophages. The infectivity of the virus produced in natural target cells of HIV-1 negatively correlated with the level of ABCA1. These findings demonstrate that Nef-dependent inhibition of ABCA1 is an essential component of the viral replication strategy and underscore the role of ABCA1 as an innate anti-HIV factor.

3.1777 Subversion of NPC1 pathway of cholesterol transport by Anaplasma phagocytophilum

Xiong, Q. and Rikihisa, Y.

Cell. Microbiol., ***14(4)***, *560-576 (2012)*

Intracellular cholesterol amounts, distribution and traffic are tightly regulated to maintain the healthy eukaryotic cell function. However, how intracellular pathogens that require cholesterol, interact with the host cholesterol homeostasis and traffic is not well understood. *Anaplasma phagocytophilum* is an obligatory intracellular and cholesterol-robbing bacterium, which causes human granulocytic anaplasmosis. Here we found that a subset of cholesterol-binding membrane protein, Niemann-Pick type C1 (NPC1)-bearing vesicles devoid of lysosomal markers were upregulated in HL-60 cells infected with *A. phagocytophilum*, and trafficked to live bacterial inclusions. The NPC1 localization to *A. phagocytophilum* inclusions was abolished by low-density lipoprotein (LDL)-derived cholesterol traffic inhibitor U18666A. Studies using NPC1 siRNA and the cell line with cholesterol traffic defect demonstrated that the NPC1 function is required for bacterial cholesterol acquisition and infection. Furthermore, trans-Golgi network-specific soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors, vesicle-associated membrane protein (VAMP4) and syntaxin 16, which are associated with NPC1 and LDL-derived cholesterol vesicular transport were recruited to *A. phagocytophilum* inclusions, and VAMP4 was required for bacteria infection. Taken together, *A. phagocytophilum* is the first example

of a pathogen that subverts the NPC1 pathway of intracellular cholesterol transport and homeostasis for bacterial inclusion membrane biogenesis and cholesterol capture.

3.1778 Isoform-specific palmitoylation of JNK regulates axonal development
Yang, G., Liu, Y., Yang, K., Liu, R., Zhu, S., Coquinco, A., Wen, W., Kojic, L. and Cynader, M.
*Cell Death and Differentiation, **19(4)**, 553-561 (2012)*

The c-jun N-terminal kinase (JNK) proteins are encoded by three genes (*Jnk1–3*), giving rise to 10 isoforms in the mammalian brain. The differential roles of JNK isoforms in neuronal cell death and development have been noticed in several pathological and physiological contexts. However, the mechanisms underlying the regulation of different JNK isoforms to fulfill their specific roles are poorly understood. Here, we report an isoform-specific regulation of JNK3 by palmitoylation, a posttranslational modification, and the involvement of JNK3 palmitoylation in axonal development and morphogenesis. Two cysteine residues at the COOH-terminus of JNK3 are required for dynamic palmitoylation, which regulates JNK3's distribution on the actin cytoskeleton. Expression of palmitoylation-deficient JNK3 increases axonal branching and the motility of axonal filopodia in cultured hippocampal neurons. The Wnt family member Wnt7a, a known modulator of axonal branching and remodelling, regulates the palmitoylation and distribution of JNK3. Palmitoylation-deficient JNK3 mimics the effect of Wnt7a application on axonal branching, whereas constitutively palmitoylated JNK3 results in reduced axonal branches and blocked Wnt7a induction. Our results demonstrate that protein palmitoylation is a novel mechanism for isoform-specific regulation of JNK3 and suggests a potential role of JNK3 palmitoylation in modulating axonal branching.

3.1779 Novel Role for Non-muscle Myosin Light Chain Kinase (MLCK) in Hyperoxia-induced Recruitment of Cytoskeletal Proteins, NADPH Oxidase Activation, and Reactive Oxygen Species Generation in Lung Endothelium
Usatyuk, P.V., Singleton, P.A., Pendyala, S., Kalari, S.K., He, D., Gorshkova, I.A., Camp, S.M., Moitra, J., Dudek, S.M., Garcia, J.G.N. and Natarajan, V.
*J. Biol. Chem., **287(12)**, 9360-9375 (2012)*

We recently demonstrated that hyperoxia (HO) activates lung endothelial cell NADPH oxidase and generates reactive oxygen species (ROS)/superoxide via Src-dependent tyrosine phosphorylation of $p47^{phox}$ and cortactin. Here, we demonstrate that the non-muscle ~214-kDa myosin light chain (MLC) kinase (nmMLCK) modulates the interaction between cortactin and $p47^{phox}$ that plays a role in the assembly and activation of endothelial NADPH oxidase. Overexpression of FLAG-tagged wild type MLCK in human pulmonary artery endothelial cells enhanced interaction and co-localization between cortactin and $p47^{phox}$ at the cell periphery and ROS production, whereas abrogation of MLCK using specific siRNA significantly inhibited the above. Furthermore, HO stimulated phosphorylation of MLC and recruitment of phosphorylated and non-phosphorylated cortactin, MLC, Src, and $p47^{phox}$ to caveolin-enriched microdomains (CEM), whereas silencing nmMLCK with siRNA blocked recruitment of these components to CEM and ROS generation. Exposure of $nmMLCK^{-/-}$ null mice to HO (72 h) reduced ROS production, lung inflammation, and pulmonary leak compared with control mice. These results suggest a novel role for nmMLCK in hyperoxia-induced recruitment of cytoskeletal proteins and NADPH oxidase components to CEM, ROS production, and lung injury.

3.1780 Therapeutic Effects of Autologous Tumor-Derived Nanovesicles on Melanoma Growth and Metastasis
Lee, E-Y., Park, K-S., Yoon, Y.J., Lee, J., Moon, H-G., Jang, S.C., Choi, K-H., Kim, Y-K. and Gho, Y.S.
*PloS One, **7(3)**, e33330 (2012)*

Cancer vaccines with optimal tumor-associated antigens show promise for anti-tumor immunotherapy. Recently, nano-sized vesicles, such as exosomes derived from tumors, were suggested as potential antigen candidates, although the total yield of exosomes is not sufficient for clinical applications. In the present study, we developed a new vaccine strategy based on nano-sized vesicles derived from primary autologous tumors. Through homogenization and sonication of tumor tissues, we achieved high yields of vesicle-bound antigens. These nanovesicles were enriched with antigenic membrane targets but lacked nuclear autoantigens. Furthermore, these nanovesicles together with adjuvant activated dendritic cells *in vitro*, and induced effective anti-tumor immune responses in both primary and metastatic melanoma mouse models. Therefore, autologous tumor-derived nanovesicles may represent a novel source of antigens with high-

level immunogenicity for use in acellular vaccines without compromising safety. Our strategy is cost-effective and can be applied to patient-specific cancer therapeutic vaccination.

3.1781 NADPH Oxidase-derived Reactive Oxygen Species Increases Expression of Monocyte Chemotactic Factor Genes in Cultured Adipocytes

Han, C.Y., Umemoto, T., Omer, M., Den Hartigh, L.J., Chiba, T., LeBoeuf, R., Buller, C.L., , Sweet, I.R., Pennathur, S., Dale Abel, E. and Chait, A.
J. Biol. Chem., ***287(13)***, *10379-10393 (2012)*

Excess glucose and free fatty acids delivered to adipose tissue causes local inflammation, which contributes to insulin resistance. Glucose and palmitate generate reactive oxygen species (ROS) in adipocytes, leading to monocyte chemotactic factor gene expression. Docosahexaenoate (DHA) has the opposite effect. In this study, we evaluated the potential sources of ROS in the presence of excess nutrients. Differentiated 3T3-L1 adipocytes were exposed to palmitate and DHA (250 μm) in either 5 or 25 mm glucose to evaluate the relative roles of mitochondrial electron transport and NADPH oxidases (NOX) as sources of ROS. Excess glucose and palmitate did not increase mitochondrial oxidative phosphorylation. However, glucose exposure increased glycolysis. Of the NOX family members, only NOX4 was expressed in adipocytes. Moreover, its activity was increased by excess glucose and palmitate and decreased by DHA. Silencing NOX4 inhibited palmitate- and glucose-stimulated ROS generation and monocyte chemotactic factor gene expression. NADPH, a substrate for NOX, and pentose phosphate pathway activity increased with glucose but not palmitate and decreased with DHA exposure. Inhibition of the pentose phosphate pathway by glucose-6-phosphate dehydrogenase inhibitors and siRNA suppressed ROS generation and monocyte chemotactic factor gene expression induced by both glucose and palmitate. Finally, both high glucose and palmitate induced NOX4 translocation into lipid rafts, effects that were blocked by DHA. Excess glucose and palmitate generate ROS via NOX4 rather than by mitochondrial oxidation in cultured adipocytes. NOX4 is regulated by both NADPH generated in the PPP and translocation of NOX4 into lipid rafts, leading to expression of monocyte chemotactic factors.

3.1782 Rab11-FIP3 is a cell cycle-regulated phosphoprotein

Collins, L.L., Simon, G., Matheson, J., Wu, C., Miller, M.C., Otani, T., Yu, X., Hayashi, S., Prekeris, R. and Gould, G.W.
BMC Biology, ***13***, *4-18 (2012)*

Background
Rab11 and its effector molecule, Rab11-FIP3 (FIP3), associate with recycling endosomes and traffic into the furrow and midbody of cells during cytokinesis. FIP3 also controls recycling endosome distribution during interphase. Here, we examine whether phosphorylation of FIP3 is involved in these activities.
Results
We identify four sites of phosphorylation of FIP3 in vivo, S-102, S-280, S-347 and S-450 and identify S-102 as a target for Cdk1-cyclin B *in vitro*. Of these, we show that S-102 is phosphorylated in metaphase and is dephosphorylated as cells enter telophase. Over-expression of FIP3-S102D increased the frequency of binucleate cells consistent with a role for this phospho-acceptor site in cytokinesis. Mutation of S-280, S-347 or S-450 or other previously identified phospho-acceptor sites (S-488, S-538, S-647 and S-648) was without effect on binucleate cell formation and did not modulate the distribution of FIP3 during the cell cycle. In an attempt to identify a functional role for FIP3 phosphorylation, we report that the change in FIP3 distribution from cytosolic to membrane-associated observed during progression from anaphase to telophase is accompanied by a concomitant dephosphorylation of FIP3. However, the phospho-acceptor sites identified here did not control this change in distribution.
Conclusions
Our data thus identify FIP3 as a cell cycle regulated phosphoprotein and suggest dephosphorylation of FIP3 accompanies its translocation from the cytosol to membranes during telophase. S102 is dephosphorylated during telophase; mutation of S102 exerts a modest effect on cytokinesis. Finally, we show that de/phosphorylation of the phospho-acceptor sites identified here (S-102, S-280, S-347 and S-450) is not required for the spatial control of recycling endosome distribution or function.

3.1783 Cell-type-specific nuclei purification from whole animals for genome-wide expression and chromatin profiling

Steiner, F.A., Talbert, P.B., Kasinathan, S. et al.
Genome Res., ***22***, *766-777 (2012)*

An understanding of developmental processes requires knowledge of transcriptional and epigenetic landscapes at the level of tissues and ultimately individual cells. However, obtaining tissue- or cell-type-specific expression and chromatin profiles for animals has been challenging. Here we describe a method for purifying nuclei from specific cell types of animal models that allows simultaneous determination of both expression and chromatin profiles. The method is based on in vivo biotin-labeling of the nuclear envelope and subsequent affinity purification of nuclei. We describe the use of the method to isolate nuclei from muscle of adult *Caenorhabditis elegans* and from mesoderm of *Drosophila melanogaster* embryos. As a case study, we determined expression and nucleosome occupancy profiles for affinity-purified nuclei from *C. elegans* muscle. We identified hundreds of genes that are specifically expressed in muscle tissues and found that these genes are depleted of nucleosomes at promoters and gene bodies in muscle relative to other tissues. This method should be universally applicable to all model systems that allow transgenesis and will make it possible to determine epigenetic and expression profiles of different tissues and cell types.

3.1784 Annexin A2 Is Involved in the Formation of Hepatitis C Virus Replication Complex on the Lipid Raft

Saxena, V., Lai, C-K., Chao, T-C., Jeng, K-S. and Lai, M.M.C.
J. Virol., ***86(8)***, *4139-4150 (2012)*

The hepatitis C virus (HCV) RNA replicates in hepatic cells by forming a replication complex on the lipid raft (detergent-resistant membrane [DRM]). Replication complex formation requires various viral nonstructural (NS) proteins as well as host cellular proteins. In our previous study (C. K. Lai, K. S. Jeng, K. Machida, and M. M. Lai, J. Virol. 82:8838–8848, 2008), we found that a cellular protein, annexin A2 (Anxa2), interacts with NS3/NS4A. Since NS3/NS4A is a membranous protein and Anxa2 is known as a lipid raft-associated scaffold protein, we postulate that Anxa2 helps in the formation of the HCV replication complex on the lipid raft. Further studies showed that Anxa2 was localized at the HCV-induced membranous web and interacted with NS4B, NS5A, and NS5B and colocalized with them in the perinuclear region. The silencing of Anxa2 decreased the formation of membranous web-like structures and viral RNA replication. Subcellular fractionation and bimolecular fluorescence complementation analysis revealed that Anxa2 was partially associated with HCV at the lipid raft enriched with phosphatidylinositol-4-phosphate (PI4P) and caveolin-2. Further, the overexpression of Anxa2 in HCV-nonsusceptible HEK293 cells caused the enrichment of HCV NS proteins in the DRM fraction and increased the colony-forming ability of the HCV replicon. Since Anxa2 is known to induce the formation of the lipid raft microdomain, we propose that Anxa2 recruits HCV NS proteins and enriches them on the lipid raft to form the HCV replication complex.

3.1785 Compartmentalization of endocannabinoids into lipid rafts in a microglial cell line devoid of caveolin-1

Rimmermann, N., Bradshaw, H.B., Kozela, E., Levy, R., Juknat, A. and Vogel, Z.
Br. J. Pharmacol., ***165(8)***, *2436-2449 (2012)*

BACKGROUND AND PURPOSE*N*-acyl ethanolamines (NAEs) and 2-arachidonoyl glycerol (2-AG) are endogenous cannabinoids and along with related lipids are synthesized on demand from membrane phospholipids. Here, we have studied the compartmentalization of NAEs and 2-AG into lipid raft fractions isolated from the caveolin-1-lacking microglial cell line BV-2, following vehicle or cannabidiol (CBD) treatment. Results were compared with those from the caveolin-1-positive F-11 cell line.
EXPERIMENTAL APPROACH BV-2 cells were incubated with CBD or vehicle. Cells were fractionated using a detergent-free continuous OptiPrep density gradient. Lipids in fractions were quantified using HPLC/MS/MS. Proteins were measured using Western blot.
KEY RESULTS BV-2 cells were devoid of caveolin-1. Lipid rafts were isolated from BV-2 cells as confirmed by co-localization with flotillin-1 and sphingomyelin. Small amounts of cannabinoid CB_1 receptors were found in lipid raft fractions. After incubation with CBD, levels and distribution in lipid rafts of 2-AG, *N*-arachidonoyl ethanolamine (AEA), and *N*-oleoyl ethanolamine (OEA) were not changed. Conversely, the levels of the saturated *N*-stearoyl ethanolamine (SEA) and *N*-palmitoyl ethanolamine (PEA) were elevated in lipid raft fractions. In whole cells with growth medium, CBD treatment increased AEA and OEA time-dependently, while levels of 2-AG, PEA and SEA did not change.
CONCLUSIONS AND IMPLICATIONS Whereas levels of 2-AG were not affected by CBD treatment, the distribution and levels of NAEs showed significant changes. Among the NAEs, the degree of acyl chain saturation predicted the compartmentalization after CBD treatment suggesting a shift in cell signalling activity.

3.1786 Apolipoprotein A-I Attenuates Palmitate-Mediated NF-κB Activation by Reducing Toll-Like Receptor-4 Recruitment into Lipid Rafts

Cheng, A.M., handa, P., Tateya, S., Schwartz, J., Tang, C., Mitra, P., Oram, J.F., Chait, A. and Kim, F.
*PloS One, **7(3)**, e33917 (2012)*

While high-density lipoprotein (HDL) is known to protect against a wide range of inflammatory stimuli, its anti-inflammatory mechanisms are not well understood. Furthermore, HDL's protective effects against saturated dietary fats have not been previously described. In this study, we used endothelial cells to demonstrate that while palmitic acid activates NF-κB signaling, apolipoprotein A–I, (apoA-I), the major protein component of HDL, attenuates palmitate-induced NF-κB activation. Further, vascular NF-κB signaling (IL-6, MCP-1, TNF-α) and macrophage markers (CD68, CD11c) induced by 24 weeks of a diabetogenic diet containing cholesterol (DDC) is reduced in human apoA-I overexpressing transgenic C57BL/6 mice compared to age-matched WT controls. Moreover, WT mice on DDC compared to a chow diet display increased gene expression of lipid raft markers such as Caveolin-1 and Flotillin-1, and inflammatory Toll-like receptors (TLRs) (TLR2, TLR4) in the vasculature. However apoA-I transgenic mice on DDC show markedly reduced expression of these genes. Finally, we show that in endothelial cells TLR4 is recruited into lipid rafts in response to palmitate, and that apoA-I prevents palmitate-induced TLR4 trafficking into lipid rafts, thereby blocking NF-κB activation. Thus, apoA-I overexpression might be a useful therapeutic tool against vascular inflammation.

3.1787 Erythropoietin Receptor Signaling Is Membrane Raft Dependent

McGraw, K.L., Fuhler, G.M., Johnson, J.O., Clark, J.A., Caceres, G.C., Sokol, L. and List, A.F.
*PloS One, **7(4)**, e34477 (2012)*

Upon erythropoietin (Epo) engagement, Epo-receptor (R) homodimerizes to activate JAK2 and Lyn, which phosphorylate STAT5. Although recent investigations have identified key negative regulators of Epo-R signaling, little is known about the role of membrane localization in controlling receptor signal fidelity. Here we show a critical role for membrane raft (MR) microdomains in creation of discrete signaling platforms essential for Epo-R signaling. Treatment of UT7 cells with Epo induced MR assembly and coalescence. Confocal microscopy showed that raft aggregates significantly increased after Epo stimulation (mean, 4.3±1.4(SE) vs. 25.6±3.2 aggregates/cell; $p \leq 0.001$), accompanied by a >3-fold increase in cluster size ($p \leq 0.001$). Raft fraction immunoblotting showed Epo-R translocation to MR after Epo stimulation and was confirmed by fluorescence microscopy in Epo stimulated UT7 cells and primary erythroid bursts. Receptor recruitment into MR was accompanied by incorporation of JAK2, Lyn, and STAT5 and their activated forms. Raft disruption by cholesterol depletion extinguished Epo induced Jak2, STAT5, Akt and MAPK phosphorylation in UT7 cells and erythroid progenitors. Furthermore, inhibition of the Rho GTPases Rac1 or RhoA blocked receptor recruitment into raft fractions, indicating a role for these GTPases in receptor trafficking. These data establish a critical role for MR in recruitment and assembly of Epo-R and signal intermediates into discrete membrane signaling units.

3.1788 Channel-Forming Activities in the Glycosomal Fraction from the Bloodstream Form of Trypanosoma brucei

Gualdron-Lopez, M., Vapola, M.H., Miinalainen, I:J., Hitunen, J.K., Michels, P.A. and Antenenkov, V.D.
*PloS One, **7(4)**, e34530 (2012)*

Background

Glycosomes are a specialized form of peroxisomes (microbodies) present in unicellular eukaryotes that belong to the Kinetoplastea order, such as *Trypanosoma* and *Leishmania* species, parasitic protists causing severe diseases of livestock and humans in subtropical and tropical countries. The organelles harbour most enzymes of the glycolytic pathway that is responsible for substrate-level ATP production in the cell. Glycolysis is essential for bloodstream-form *Trypanosoma brucei* and enzymes comprising this pathway have been validated as drug targets. Glycosomes are surrounded by a single membrane. How glycolytic metabolites are transported across the glycosomal membrane is unclear.

Methods/Principal Findings

We hypothesized that glycosomal membrane, similarly to membranes of yeast and mammalian peroxisomes, contains channel-forming proteins involved in the selective transfer of metabolites. To verify this prediction, we isolated a glycosomal fraction from bloodstream-form *T.brucei* and reconstituted solubilized membrane proteins into planar lipid bilayers. The electrophysiological characteristics of the channels were studied using multiple channel recording and single channel analysis. Three main channel-forming activities were detected with current amplitudes 70–80 pA, 20–25 pA, and 8–11 pA, respectively

(holding potential +10 mV and 3.0 M KCl as an electrolyte). All channels were in fully open state in a range of voltages ±150 mV and showed no sub-conductance transitions. The channel with current amplitude 20–25 pA is anion-selective (P_{K+}/P_{Cl-}~0.31), while the other two types of channels are slightly selective for cations (P_{K+}/P_{Cl-} ratios ~1.15 and ~1.27 for the high- and low-conductance channels, respectively). The anion-selective channel showed an intrinsic current rectification that may suggest a functional asymmetry of the channel's pore.

Conclusions/Significance

These results indicate that the membrane of glycosomes apparently contains several types of pore-forming channels connecting the glycosomal lumen and the cytosol.

3.1789 NGF Causes TrkA to Specifically Attract Microtubules to Lipid Rafts

Pryor, S., McCaffrey, G., Young, L.R. and Grimes, M.L.
*PloS One, **7(4)**, e35163 (2012)*

Membrane protein sorting is mediated by interactions between proteins and lipids. One mechanism that contributes to sorting involves patches of lipids, termed lipid rafts, which are different from their surroundings in lipid and protein composition. Although the nerve growth factor (NGF) receptors, TrkA and $p75^{NTR}$ collaborate with each other at the plasma membrane to bind NGF, these two receptors are endocytosed separately and activate different cellular responses. We hypothesized that receptor localization in membrane rafts may play a role in endocytic sorting. TrkA and $p75^{NTR}$ both reside in detergent-resistant membranes (DRMs), yet they responded differently to a variety of conditions. The ganglioside, GM1, caused increased association of NGF, TrkA, and microtubules with DRMs, but a decrease in $p75^{NTR}$. When microtubules were induced to polymerize and attach to DRMs by *in vitro* reactions, TrkA, but not $p75^{NTR}$, was bound to microtubules in DRMs and in a detergent-resistant endosomal fraction. NGF enhanced the interaction between TrkA and microtubules in DRMs, yet tyrosine phosphorylated TrkA was entirely absent in DRMs under conditions where activated TrkA was detected in detergent-sensitive membranes and endosomes. These data indicate that TrkA and $p75^{NTR}$ partition into membrane rafts by different mechanisms, and that the fraction of TrkA that associates with DRMs is internalized but does not directly form signaling endosomes. Rather, by attracting microtubules to lipid rafts, TrkA may mediate other processes such as axon guidance.

3.1790 Requirement of translocated lysosomal V1 H+-ATPase for activation of membrane acid sphingomyelinase and raft clustering in coronary endothelial cells

Xu, M., Xia, M., Li, X-X., Han, W-Q., Boini, K.M., Zhang, F., Zhang, Y., Ritter, J.K. and Li, P-L.
*Mol. Biol. Cell, **23**, 1546-1557 (2012)*

Acid sphingomyelinase (ASM) mediates the formation of membrane raft (MR) redox signalosomes in a process that depends on a local acid microenvironment in coronary arterial endothelial cells (CAECs). However, it is not known how this local acid microenvironment is formed and maintained. The present study hypothesized that lysosomal V1 H^+-ATPase provides a hospitable acid microenvironment for activation of ASM when lysosomes traffic and fuse into the cell membrane. Confocal microscopy showed that local pH change significantly affected MRs, with more fluorescent patches under low pH. Correspondingly, the ASM product, ceramide, increased locally in the cell membrane. Electron spin resonance assay showed that local pH increase significantly inhibited NADPH oxidase–mediated production of $O_2^{-\cdot}$ in CAECs. Direct confocal microscopy demonstrated that Fas ligand resulted in localized areas of decreased pH around CAEC membranes. The inhibitors of both lysosomal fusion and H^+-ATPase apparently attenuated FasL-caused pH decrease. V1 H^+-ATPase accumulation and activity on cell membranes were substantially suppressed by the inhibitors of lysosomal fusion or H^+-ATPase. These results provide the first direct evidence that translocated lysosomal V1 H^+-ATPase critically contributes to the formation of local acid microenvironment to facilitate activation of ASM and consequent MR aggregation, forming MR redox signalosomes and mediating redox signaling in CAECs.

3.1791 Host Acyl Coenzyme A Binding Protein Regulates Replication Complex Assembly and Activity of a Positive-Strand RNA Virus

Zhang, J., Diaz, A., Mao, L., Ahlquist, P and Wang, X.
*J. Virol., **86(9)**, 5110-5121 (2012)*

All positive-strand RNA viruses reorganize host intracellular membranes to assemble their replication complexes. Similarly, brome mosaic virus (BMV) induces two alternate forms of membrane-bound RNA

replication complexes: vesicular spherules and stacks of appressed double-membrane layers. The mechanisms by which these membrane rearrangements are induced, however, remain unclear. We report here that host ACB1-encoded acyl coenzyme A (acyl-CoA) binding protein (ACBP) is required for the assembly and activity of both BMV RNA replication complexes. ACBP is highly conserved among eukaryotes, specifically binds to long-chain fatty acyl-CoA, and promotes general lipid synthesis. Deleting ACB1 inhibited BMV RNA replication up to 30-fold and resulted in formation of spherules that were ~50% smaller but ~4-fold more abundant than those in wild-type (wt) cells, consistent with the idea that BMV 1a invaginates and maintains viral spherules by coating the inner spherule membrane. Furthermore, smaller and more frequent spherules were preferentially formed under conditions that induce layer formation in wt cells. Conversely, cellular karmella structures, which are arrays of endoplasmic reticulum (ER) membranes formed upon overexpression of certain cellular ER membrane proteins, were formed normally, indicating a selective inhibition of 1a-induced membrane rearrangements. Restoring altered lipid composition largely complemented the BMV RNA replication defect, suggesting that ACBP was required for maintaining lipid homeostasis. Smaller and more frequent spherules are also induced by 1a mutants with specific substitutions in a membrane-anchoring amphipathic α-helix, implying that the 1a-lipid interactions play critical roles in viral replication complex assembly.

3.1792 Lysosome fusion to the cell membrane is mediated by the dysferlin C2A domain in coronary arterial endothelial cells

Han, W-Q., Xia, M., Xu, M., Boini, K.M., Ritter, J.K., Li, N-J. and Li, P-L.
J. Cell Sci., ***125****, 1225-1234 (2012)*

Dysferlin has recently been reported to participate in cell membrane repair in muscle and other cells through lysosome fusion. Given that lysosome fusion is a crucial mechanism that leads to membrane raft clustering, the present study attempted to determine whether dysferlin is involved in this process and its related signalling, and explores the mechanism underlying dysferlin-mediated lysosome fusion in bovine coronary arterial endothelial cells (CAECs). We found that dysferlin is clustered in membrane raft macrodomains after Fas Ligand (FasL) stimulation as detected by confocal microscopy and membrane fraction flotation. Small-interfering RNA targeted to dysferlin prevented membrane raft clustering. Furthermore, the translocation of acid sphingomyelinase (ASMase) to membrane raft clusters, whereby local ASMase activation and ceramide production – an important step that mediates membrane raft clustering – was attenuated. Functionally, silencing of the dysferlin gene reversed FasL-induced impairment of endothelium-dependent vasodilation in isolated small coronary arteries. By monitoring fluorescence quenching or dequenching, silencing of the dysferlin gene was found to almost completely block lysosome fusion to plasma membrane upon FasL stimulation. Further studies to block C2A binding and silencing of *AHNAK* (a dysferlin C2A domain binding partner), showed that the dysferlin C2A domain is required for FasL-induced lysosome fusion to the cell membrane, ASMase translocation and membrane raft clustering. We conclude that dysferlin determines lysosome fusion to the plasma membrane through its C2A domain and it is therefore implicated in membrane-raft-mediated signaling and regulation of endothelial function in coronary circulation.

3.1793 Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes

Tauro, B.J., Greening, D.W., Mathias, R.A., Ji, H., Mathivanan, S., Scott, A.M. and Simpson, R.J.
Methods, ***56****, 293-304 (2012)*

Exosomes are 40–100 nm extracellular vesicles that are released from a multitude of cell types, and perform diverse cellular functions including intercellular communication, antigen presentation, and transfer of oncogenic proteins as well as mRNA and miRNA. Exosomes have been purified from biological fluids and *in vitro* cell cultures using a variety of strategies and techniques. However, all preparations invariably contain varying proportions of other membranous vesicles that co-purify with exosomes such as shed microvesicles and apoptotic blebs. Using the colorectal cancer cell line LIM1863 as a cell model, in this study we performed a comprehensive evaluation of current methods used for exosome isolation including ultracentrifugation (UC-Exos), OptiPrep™ density-based separation (DG-Exos), and immunoaffinity capture using anti-EpCAM coated magnetic beads (IAC-Exos). Notably, all isolations contained 40–100 nm vesicles, and were positive for exosome markers (Alix, TSG101, HSP70) based on electron microscopy and Western blotting. We employed a proteomic approach to profile the protein composition of exosomes, and label-free spectral counting to evaluate the effectiveness of each method. Based on the number of MS/MS spectra identified for exosome markers and proteins associated with their biogenesis, trafficking, and release, we found IAC-Exos to be the most effective method to

isolate exosomes. For example, Alix, TSG101, CD9 and CD81 were significantly higher (at least 2-fold) in IAC-Exos, compared to UG-Exos and DG-Exos. Application of immunoaffinity capture has enabled the identification of proteins including the ESCRT-III component VPS32C/CHMP4C, and the SNARE synaptobrevin 2 (VAMP2) in exosomes for the first time. Additionally, several cancer-related proteins were identified in IAC-Exos including various ephrins (EFNB1, EFNB2) and Eph receptors (EPHA2‑8, EPHB1‑4), and components involved in Wnt (CTNNB1, TNIK) and Ras (CRK, GRB2) signalling.

3.1794 Accumulation of an Antidepressant in Vesiculogenic Membranes of Yeast Cells Triggers Autophagy
Chen, J., Korostyshevsky, D., Lee, S. and Perlstein, E.O.
*PloS One, **7(4)**, e34024 (2012)*

Many antidepressants are cationic amphipaths, which spontaneously accumulate in natural or reconstituted membranes in the absence of their specific protein targets. However, the clinical relevance of cellular membrane accumulation by antidepressants in the human brain is unknown and hotly debated. Here we take a novel, evolutionarily informed approach to studying the effects of the selective-serotonin reuptake inhibitor sertraline/Zoloft® on cell physiology in the model eukaryote *Saccharomyces cerevisiae* (budding yeast), which lacks a serotonin transporter entirely. We biochemically and pharmacologically characterized cellular uptake and subcellular distribution of radiolabeled sertraline, and in parallel performed a quantitative ultrastructural analysis of organellar membrane homeostasis in untreated vs. sertraline-treated cells. These experiments have revealed that sertraline enters yeast cells and then reshapes vesiculogenic membranes by a complex process. Internalization of the neutral species proceeds by simple diffusion, is accelerated by proton motive forces generated by the vacuolar H^+-ATPase, but is counteracted by energy-dependent xenobiotic efflux pumps. At equilibrium, a small fraction (10–15%) of reprotonated sertraline is soluble while the bulk (90–85%) partitions into organellar membranes by adsorption to interfacial anionic sites or by intercalation into the hydrophobic phase of the bilayer. Asymmetric accumulation of sertraline in vesiculogenic membranes leads to local membrane curvature stresses that trigger an adaptive autophagic response. In mutants with altered clathrin function, this adaptive response is associated with increased lipid droplet formation. Our data not only support the notion of a serotonin transporter-independent component of antidepressant function, but also enable a conceptual framework for characterizing the physiological states associated with chronic but not acute antidepressant administration in a model eukaryote.

3.1795 Proteomic characterisation of endoplasmic reticulum-derived protein bodies in tobacco leaves
Joseph, M., Ludevid, M.D., Torrent, M., Rofidal, V., Tauzin, M., Rossignol, M. and Peltier, J-B.
*BMC Plant Biol., **12**, 36 (2012)*

Background
The N-terminal proline-rich domain (Zera) of the maize storage protein γ-zein, is able to induce the formation of endoplasmic reticulum (ER)-derived protein bodies (PBs) when fused to proteins of interest. This encapsulation enables a recombinant fused protein to escape from degradation and facilitates its recovery from plant biomass by gradient purification. The aim of the present work was to evaluate if induced PBs encapsulate additional proteins jointly with the recombinant protein. The exhaustive analysis of protein composition of PBs is expected to facilitate a better understanding of PB formation and the optimization of recombinant protein purification approaches from these organelles.
Results
We analysed the proteome of PBs induced in *Nicotiana benthamiana* leaves by transient transformation with Zera fused to a fluorescent marker protein (DsRed). Intact PBs with their surrounding ER-membrane were isolated on iodixanol based density gradients and their integrity verified by confocal and electron microscopy. SDS-PAGE analysis of isolated PBs showed that Zera-DsRed accounted for around 85% of PB proteins in term of abundance. Differential extraction of PBs was performed for in-depth analysis of their proteome and structure. Besides Zera-DsRed, 195 additional proteins were identified including a broad range of proteins resident or trafficking through the ER and recruited within the Zera-DsRed polymer.
Conclusions
This study indicates that Zera-protein fusion is still the major protein component of the new formed organelle in tobacco leaves. The analysis also reveals the presence of an unexpected diversity of proteins in PBs derived from both the insoluble Zera-DsRed polymer formation, including ER-resident and secretory proteins, and a secretory stress response induced most likely by the recombinant protein overloading.

Knowledge of PBs protein composition is likely to be useful to optimize downstream purification of recombinant proteins in molecular farming applications.

3.1796 Targeted Disruption of Core 1 β1,3-galactosyltransferase (C1galt1) Induces Apical Endocytic Trafficking in Human Corneal Keratinocytes

Guzman-Aranguez, A., Woodward, A.M., Pintor, J. and Argüeso, P.
*PloS One, **7(5)**, e36628 (2012)*

Background
Exposed mucosal surfaces limit constitutive endocytosis under physiological conditions to prevent uptake of macromolecules and pathogens and, therefore, cellular damage. It is now accepted that cell surface mucins, a group of high molecular weight glycoproteins on the epithelial glycocalyx, defined by their extensive O-glycosylation, play a major role in maintaining barrier function in these surfaces, but the precise mechanisms are unclear.
Methodology/Principal Findings
In this work, we utilized a stable tetracycline-inducible RNA interfering system targeting the core 1 ß1,3-galactosyltransferase (C1galt1 or T-synthase), a critical galactosyltransferase required for the synthesis of core 1 O-glycans, to explore the role of mucin-type carbohydrates in apical endocytic trafficking in human corneal keratinocytes. Using cell surface biotinylation and subcellular fractionation, we found increased accumulation of plasma membrane protein in endosomes after C1galt1 depletion. Confocal laser scanning microscopy and fluorometry revealed increased translocation of negatively charged fluorescent nanospheres after C1galt1 knockdown sustained by an active transport process and largely independent of apical intercellular junctions. Internalization of nanospheres could be blocked by dynasore, nocodazole, chlorpromazine, and hyperosmotic sucrose, suggesting a mechanism for clathrin-coated pit budding and vesicular trafficking. This possibility was supported by experiments showing nanosphere colocalization with clathrin heavy chain in the cytoplasm.
Conclusions/Significance
Together, the data suggest that core 1 O-glycans contribute to maintenance of apical barrier function on exposed mucosal surfaces by preventing clathrin-mediated endocytosis.

3.1797 Novel Role for Proteinase-activated Receptor 2 (PAR2) in Membrane Trafficking of Proteinase-activated Receptor 4 (PAR4)

Cunningham, M.R., McIntosh, K.A., Pediani, J.D., Robben, J., Cooke, A.E., Nilsson, M., Gould, G.W.., Mundell, S., Milligan, G. and Plevin, R.
*J. Biol. Chem., **287(20)**, 16656-16669 (2012)*

Proteinase-activated receptors 4 (PAR_4) is a class A G protein-coupled receptor (GPCR) recognized through the ability of serine proteases such as thrombin and trypsin to mediate receptor activation. Due to the irreversible nature of activation, a fresh supply of receptor is required to be mobilized to the cell surface for responsiveness to agonist to be sustained. Unlike other PAR subtypes, the mechanisms regulating receptor trafficking of PAR_4 remain unknown. Here, we report novel features of the intracellular trafficking of PAR_4 to the plasma membrane. PAR_4 was poorly expressed at the plasma membrane and largely retained in the endoplasmic reticulum (ER) in a complex with the COPI protein subunit β-COP1. Analysis of the PAR_4 protein sequence identified an arginine-based (RXR) ER retention sequence located within intracellular loop-2 ($R^{183}AR \rightarrow A^{183}AA$), mutation of which allowed efficient membrane delivery of PAR_4. Interestingly, co-expression with PAR_2 facilitated plasma membrane delivery of PAR_4, an effect produced through disruption of β-COP1 binding and facilitation of interaction with the chaperone protein 14-3-3ζ. Intermolecular FRET studies confirmed heterodimerization between PAR_2 and PAR_4. PAR_2 also enhanced glycosylation of PAR_4 and activation of PAR_4 signaling. Our results identify a novel regulatory role for PAR_2 in the anterograde traffic of PAR_4. PAR_2 was shown to both facilitate and abrogate protein interactions with PAR_4, impacting upon receptor localization and cell signal transduction. This work is likely to impact markedly upon the understanding of the receptor pharmacology of PAR_4 in normal physiology and disease.

3.1798 Membrane-bound Trafficking Regulates Nuclear Transport of Integral Epidermal Growth Factor Receptor (EGFR) and ErbB-2

Wang, Y-N., Lee, H-H., Lee, H-J., Du, Y., Yamaguchi, H. ansd Hung, M-C.
*J. Biol. Chem., **287(20)**, 16869-16879 (2012)*

Nuclear localization of multiple receptor-tyrosine kinases (RTKs), such as EGF receptor (EGFR), ErbB-2, FGF receptor (FGFR), and many others, has been reported by several groups. We previously showed that cell surface EGFR is trafficked to the nucleus through a retrograde pathway from the Golgi to the endoplasmic reticulum (ER) and that EGFR is then translocated to the inner nuclear membrane (INM) through the INTERNET (integral trafficking from the ER to the nuclear envelope transport) pathway. However, the nuclear trafficking mechanisms of other membrane RTKs, apart from EGFR, remain unclear. The purpose of this study was to compare the nuclear transport of EGFR family proteins with that of FGFR-1. Interestingly, we found that digitonin permeabilization, which selectively releases soluble nuclear transporters from the cytoplasm and has been shown to inhibit nuclear transport of FGFR-1, had no effects on EGFR nuclear transport, raising the possibility that EGFR and FGFR-1 use different pathways to be translocated into the nucleus. Using the subnuclear fractionation assay, we further demonstrated that biotinylated cell surface ErbB-2, but not FGFR-1, is targeted to the INM, associating with Sec61β in the INM, similar to the nuclear trafficking of EGFR. Thus, ErbB-2, but not FGFR-1, shows a similar trafficking pathway to EGFR for translocation to the nucleus, indicating that at least two different pathways of nuclear transport exist for cell surface receptors. This finding provides a new direction for investigating the trafficking mechanisms of various nuclear RTKs.

3.1799 Raft coalescence and Fc γ RIIA activation upon sphingomyelin clustering induced by lysenin
Kulma, M., Kwiatkowska, K. and Sobota, A.
*Cellular Signalling, **24**, 1641-1647 (2012)*

Activation of immunoreceptor Fc γ RIIA by cross-linking with antibodies is accompanied by coalescence of sphingolipid/cholesterol-rich membrane rafts leading to the formation of signaling platforms of the receptor. In this report we examined whether clustering of the raft lipid sphingomyelin can reciprocally induce partition of Fc γ RIIA to rafts. To induce sphingomyelin clustering, cells were exposed to non-lytic concentrations of GST-lysenin which specifically recognizes sphingomyelin. The lysenin/sphingomyelin complexes formed microscale assemblies composed of GST-lysenin oligomers engaging sphingomyelin of rafts. Upon sphingomyelin clustering, non-cross-linked Fc γ RIIA associated with raft-derived detergent-resistant membrane fractions as revealed by density gradient centrifugation. Pretreatment of cells with GST-lysenin also increased the size of detergent-insoluble molecular complexes of activated Fc γ RIIA. Sphingomyelin clustering triggered tyrosine phosphorylation of the receptor and its accompanying proteins, Cbl and NTAL, in the absence of receptor ligands and enhanced phosphorylation of these proteins in the ligand presence. These data indicate that clustering of plasma membrane sphingomyelin induces coalescence of rafts and triggers signaling events analogous to those caused by Fc γ RIIA activation.

3.1800 Proteomic Analysis Of The Lysosomal Fraction In Trabecular Meshwork Cells Subjected To Chronic Oxidative Stress
Porter, K.M., Jeyabalan, N., Skiba, N.P., Epstein, D.L. and Liton, P.B.
*Invest. Ophthalmol. Vis. Sci., **53**, E-abstract 3248 (2012)*

Purpose: Previous work in our laboratory reported impaired lysosomal function in trabecular meshwork (TM) cells subjected to chronic oxidative stress. Here we compare the proteomic composition of isolated lysosomes from TM cells grown under physiological and oxidative stress conditions.
Methods: Confluent cultures of porcine TM cells were grown for two weeks under physiological (5% O2) and chronic oxidative stress (40% O2) conditions. The lysosomal fraction was isolated by ultracentrifugation and cell fractioning in **OptiPrep** gradients. Lysosomal proteins were separated in SDS-PAGE gels, followed by in-gel trypsin digestion. The tryptic-digested peptides were identified by peptide mass finger printing analyses and tandem mass spectrometry. Protein expression levels were quantified by WB analysis using specific antibodies against LAMP1, Rab7, CTSB, CTSD, and CD63. Cathepsin activities were assayed using fluorogenic substrates (z-FR-AMC, z-RR-AMC, z-GPR-AMC, z-VVR-AMC, CTSD/E substrate).
Results: Using an acceptance criteria for protein identification of at least two peptides with confidence interval percentage over 95%, we identified a total of 83 proteins. These included lysosomal matrix proteins and lysosomal enzymes, structural glycoproteins (LAMP1, LAMP2, LIMP2, CD63), proteins involved in translocation and lysosomal acidification (vacuolar H+ATPases), and membrane trafficking proteins. The most remarkable difference was the absence of CD63 and lysosomal acid phosphatase peptides in the lysosomal fraction from TM cells grown at 40% O2. Confirming our results obtained using whole lysates, the lysosomal fraction from oxidatively stressed cultures displayed increased LAMP1, CTSB and CTSD protein levels. However, no increased in cathepsin activities were observed. Furthermore,

the proteolytic processing of CSTB from single-chain to double-chain was significantly blocked in the stressed cultures.
Conclusions: Here we have characterized for the first time the proteome composition of lysosomes from oxidatively stressed TM cells. Our results indicate that chronic exposure to oxidative stress induces changes in the lysosomal proteomic composition and defective lysosomal function. Since the lysosomal system is responsible for the turnover of cellular organelles and degradation of phagocytosed material, diminished lysosomal activity may lead to progressive failure of the cellular TM function with age and contribute to the pathogenesis of primary open angle glaucoma.

3.1801 DHA Increases Outflow Resistance In Porcine Organ Cultures

Giovingo, M., McCarty, R.D., Beverley, R., Nolan, M., Grybauskas, A., Burdi, R.A., Wagner, E. and Knepper, P.A.

Invest. Ophthalmol. Vis. Sci., ***53***, *E-abstract 2494 (2012)*

Purpose: Caveolae and lipid rafts are highly specialized microdomains of the endothelial plasma membrane which function in signal transduction, endocytosis and transcellular fluid dynamics. Caveolin, a 21 kD protein, is an intergal component in the activity of endothelial cells. Increasing evidence suggests that omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) alter the basic properties of caveolae in endothelia and their function. The purpose of this study was to determine whether DHA changes outflow resistance in porcine anterior segment organ culture and profiles of lipid raft proteins of the trabecular meshwork (TM).
Methods: Anterior segments of porcine eyes were placed in organ culture and perfused with Dulbecco's modified eagle medium (DMEM). Flow rates were measured in eyes treated with DHA, cavtratin (a synthetic cell permeable peptide corresponding to amino acids 82 through 101 of caveolin: DGIWKASFTIFTVTKYWFYR which is also known as caveolin scaffolding domain), or DMEM. Perturbation of lipid raft containing proteins was assessed by **Optiprep** density gradient and western blot analysis of dissected TM.
Results: Flow rates are expressed as percentage change (+/-) from the baseline. Rates significantly decreased from baseline in DHA and cavtratin infused eyes.Analysis of lipid raft containing proteins of DHA and primary cultures of TM cells revealed a decrease in caveolin-1 in comparison with controls.

time	*6uM DHA*	*P value DHA vs DMEM*	*P value DHA vs CAV*	*100 ng CAV*	*P value CAV vs DMEM*	*DME*
30 min	7.7 ± 4.5	*i*	*i*	11.4 ± 6.2	i	46.8
1 hr	2.5 ± 2.5	*i*	*i*	13.1 ± 4.9	i	29.9
3 hr	-10.3 ± 8.1	*.04*	*i*	5.9 ± 3.6	i	17.6
4.5 hr	-12.8 ± 9.0	*.03*	*.04*	4.6 ± 2.7	i	16.7
6 hr	-17.0 ± 7.7	*.01*	*.04*	-1.0 ± 3.2	i	14.4
12 hr	-24.9 ± 11.5	*.03*	*i*	-18.7 ± 3.8	*.03*	1.5
Day1	-32.4 ± 11.8	*.05*	*i*	-36.3 ± 4.4	*.007*	-11.8
Day 2	-39.8 ± 9.1	*i*	*i*	-45.3 ± 4.5	*.009*	-19.1
Day 3	-38.0 ± 8.4	*i*	*i*	-48.3 ± 4.4	*.004*	-21.5

Conclusions: Infusion of DHA and cavtratin significantly decreased outflow facility and was time-dependent. DHA alters the lipid raft and caveolae microdomain, thereby influencing outflow facility in organ perfusion culture.

3.1802 Disruption of the P4-ATPase Aminophospholipid Flippase Atp8a2 Gene Suggests a Role for Phosphatidylserine in Photoreceptor Outer Segments

Coleman, J.A., Djajadi, H.R., Molday, L.L. and Molday, R.S.

Purpose: ATP8A2 is a P_4-ATPase which transports phosphatidylserine (PS) from the exocytoplasmic to the cytoplasmic side of photoreceptor outer segment (OS) disc membranes to generate and maintain PS asymmetry. The goal of this study was to determine the importance of ATP8A2 in photoreceptors and other cell types by the targeted disruption of the *atp8a2* gene in mice.
Methods: Exons 11 - 13 of *atp8a2* were replaced using a targeting construct containing a neomycin cassette in hybrid 129 SvEv and C57BL/6 ES cells. Retina membranes were prepared using the **Optiprep** method. Lipids were separated by thin layer chromatography and quantified by phosphorus assays. Retinoids were analyzed by HPLC. Antibodies specific to OS proteins were used to detect their expression and localization by western blotting and immunofluorescence microscopy. Outer nuclear layer (ONL) thickness was measured by DAPI labeling using the optic nerve as a reference. Ultrathin sections of glutaraldehyde-fixed resin-embedded retina were analyzed by transmission electron microscopy (TEM).
Results: *Atp8a2* knockout mice are smaller and catatonic compared to wild type littermates. RT-PCR, western blotting, and immunocytochemistry confirmed the absence of ATP8A2 in the retinas of the *atp8a2* knockout mice. CDC50A, the β-subunit of ATP8A2, was present at significantly lower levels. Opsin, CNGA1, ABCA4 and other outer segment (OS) proteins were reduced by approximately 50%, but correctly localized to the OS. The OS layer was approximately 50% shorter. OS contained significantly less phosphatidylserine and higher levels of phosphatidylcholine (PC). The number of photoreceptors was reduced by 15% at P23 with no further reduction evident at P50. The ultrastructure of the OS as visualized by EM appeared normal. Spectrophotometry and HPLC measurements revealed that rhodopsin and retinoid levels are 66% lower in the knockout retina.
Conclusions: Analysis of *atp8a2* knockout mice suggests that PS asymmetry and composition plays a role in photoreceptor OS morphogenesis possibly associated with the trafficking of proteins to the OS. PS asymmetry may also play a crucial role in protein trafficking in other neurons.

3.1803 Fas Ligand Enhances Malignant Behavior of Tumor Cells through Interaction with Met, Hepatocyte Growth Factor Receptor, in Lipid Rafts
Lin, H-C., Lai, P-Y., Lin, Y-p., Huang, J-H. and Yang, B-C.
*J. Biol. Chem., **287(24)**, 20664-20673 (2012)*

Many late-stage cancer cells express Fas ligand (FasL) and show high malignancy with metastatic potential. We report here a novel signaling mechanism for FasL that hijacks the Met signal pathway to promote tumor metastasis. FasL-expressing human tumor cells express a significant amount of phosphorylated Met. The down-regulation of FasL in these cells led to decreased Met activity and reduced cell motility. Ectopic expression of human FasL in NIH3T3 cells significantly stimulated their migration and invasion. The inhibition of Met and Stat3 activities reverted the FasL-associated phenotype. Notably, FasL variants activated the Met pathway, even though most of their intracellular domain or Fas binding sites were deleted. FasL interacted with Met through the FasL(105–130) extracellular region in lipid rafts, which consequently led to Met activation. Knocking down Met gene expression by RNAi technology reverted the FasL-associated motility to basal levels. Furthermore, treatment with synthetic peptides corresponding to FasL(117–126) significantly reduced the FasL/Met interaction, Met phosphorylation, and cell motility of $FasL^+$ transfectants and tumor cells. Finally, the transfectants of truncated FasL showed strong anchorage-independent growth and lung metastasis potential in null mice. Collectively, our results establish the FasL-Met-Stat3 signaling pathway and explains the metastatic phenotype of FasL-expressing tumors.

3.1804 Lecithin:Cholesterol Acyltransferase Deficiency Protects against Cholesterol-induced Hepatic Endoplasmic Reticulum Stress in Mice
Hager, L., Li, L., Pun, H., Liu, L., Hossain, M.A., Maguire, G.F., Naples, M., Baker, C., Magomedova, L., Tam, J., Adeli, K., Cummins, C.L., Connelly, P.W. and Ng, D.S.
*J. Biol. Chem., **287(24)**, 20775-20768 (2012)*

We recently reported that lecithin:cholesterol acyltransferase (LCAT) knock-out mice, particularly in the LDL receptor knock-out background, are hypersensitive to insulin and resistant to high fat diet-induced insulin resistance (IR) and obesity. We demonstrated that chow-fed *Ldlr*−/−*xLcat*+/+ mice have elevated hepatic endoplasmic reticulum (ER) stress, which promotes IR, compared with wild-type controls, and this effect is normalized in *Ldlr*−/−*xLcat*−/− mice. In the present study, we tested the hypothesis that hepatic ER cholesterol metabolism differentially regulates ER stress using these models. We observed that the *Ldlr*−/−*xLcat*+/+ mice accumulate excess hepatic total and ER cholesterol primarily attributed to increased

reuptake of biliary cholesterol as we observed reduced biliary cholesterol in conjunction with decreased hepatic *Abcg5*/*g8* mRNA, increased *Npc1l1* mRNA, and decreased *Hmgr* mRNA and nuclear SREBP2 protein. Intestinal NPC1L1 protein was induced. Expression of these genes was reversed in the *Ldlr*−/−*xLcat*−/− mice, accounting for the normalization of total and ER cholesterol and ER stress. Upon feeding a 2% high cholesterol diet (HCD), *Ldlr*−/−*xLcat*−/− mice accumulated a similar amount of total hepatic cholesterol compared with the *Ldlr*−/−*xLcat*+/+ mice, but the hepatic ER cholesterol levels remained low in conjunction with being protected from HCD-induced ER stress and IR. Hepatic ER stress correlates strongly with hepatic ER free cholesterol but poorly with hepatic tissue free cholesterol. The unexpectedly low ER cholesterol seen in HCD-fed *Ldlr*−/−*xLcat*−/− mice was attributable to a coordinated marked up-regulation of ACAT2 and suppressed SREBP2 processing. Thus, factors influencing the accumulation of ER cholesterol may be important for the development of hepatic insulin resistance.

3.1805 Activation of Myeloid Cell-Specific Adhesion Class G Protein-Coupled Receptor EMR2 via Ligation-Induced Translocation and Interaction of Receptor Subunits in Lipid Raft Microdomains
Huang, Y-S., Chiang, N-Y., Hu, C-H., Hsiao, C-C., Cheng, K-F., Tsai, W-P., Yona, S., Stavey, M., Gordon, S., Chang, XG-W. and Lin, H-H.
Mol. Cell. Biol., ***32(8)***, *1408-1420 (2012)*

The adhesion class G protein-coupled receptors (adhesion-GPCRs) play important roles in diverse biological processes ranging from immunoregulation to tissue polarity, angiogenesis, and brain development. These receptors are uniquely modified by self-catalytic cleavage at a highly conserved GPCR proteolysis site (GPS) dissecting the receptor into an extracellular subunit (α) and a seven-pass transmembrane subunit (β) with cellular adhesion and signaling functions, respectively. Using the myeloid cell-restricted EMR2 receptor as a paradigm, we exam the mechanistic relevance of the subunit interaction and demonstrate a critical role for GPS autoproteolysis in mediating receptor signaling and cell activation. Interestingly, two distinct receptor complexes are identified as a result of GPS proteolysis: one consisting of a noncovalent α-β heterodimer and the other comprising two completely independent receptor subunits which distribute differentially in membrane raft microdomains. Finally, we show that receptor ligation induces subunit translocation and colocalization within lipid rafts, leading to receptor signaling and inflammatory cytokine production by macrophages. Our present data resolve earlier conflicting results and provide a new mechanism of receptor signaling, as well as providing a paradigm for signal transduction within the adhesion-GPCR family.

3.1806 Vaccinia Virus A6 Is Essential for Virion Membrane Biogenesis and Localization of Virion Membrane Proteins to Sites of Virion Assembly
Meng, X., Embry, A., Rose, L., Yan, B., Xu, C. and Xiang, Y.
J. Virol., ***86(10)***, *5603-5613 (2012)*

Poxvirus acquires its primary envelope through a process that is distinct from those of other enveloped viruses. The molecular mechanism of this process is poorly understood, but several poxvirus proteins essential for the process have been identified in studies of vaccinia virus (VACV), the prototypical poxvirus. Previously, we identified VACV A6 as an essential factor for virion morphogenesis by studying a temperature-sensitive mutant with a lesion in A6. Here, we further studied A6 by constructing and characterizing an inducible virus (iA6) that could more stringently repress A6 expression. When A6 expression was induced by the inducer isopropyl-β-d-thiogalactoside (IPTG), iA6 replicated normally, and membrane proteins of mature virions (MVs) predominantly localized in viral factories where virions were assembled. However, when A6 expression was repressed, electron microscopy of infected cells showed the accumulation of large viroplasm inclusions containing virion core proteins but no viral membranes. Immunofluorescence and cell fractionation studies showed that the major MV membrane proteins A13, A14, D8, and H3 did not localize to viral factories but instead accumulated in the secretory compartments, including the endoplasmic reticulum. Overall, our results show that A6 is an additional VACV protein that participates in an early step of virion membrane biogenesis. Furthermore, A6 is required for MV membrane protein localization to sites of virion assembly, suggesting that MV membrane proteins or precursors of MV membranes are trafficked to sites of virion assembly through an active, virus-mediated process that requires A6.

3.1807 Critical Comparison of Multidimensional Separation Methods for Increasing Protein Expression Coverage
Antberg, L., Cifani, P., Sandin, M., Levander, F. and James, P.

J. Proteome Res., ***11(5)***, *2644-2652 (2012)*

We present a comparison of two-dimensional separation methods and how they affect the degree of coverage of protein expression in complex mixtures. We investigated the relative merits of various protein and peptide separations prior to acidic reversed-phase chromatography directly coupled to an ion trap mass spectrometer. The first dimensions investigated were density gradient organelle fractionation of cell extracts, 1D SDS-PAGE protein separation followed by digestion by trypsin or GluC proteases, strong cation exchange chromatography, and off-gel isoelectric focusing of tryptic peptides. The number of fractions from each first dimension and the total data accumulation RP-HPLC–MS/MS time was kept constant and the experiments were run in triplicate. We find that the most critical parameters are the data accumulation time, which defines the level of under-sampling and the avoidance of peptides from high expression level proteins eluting over the entire gradient.

3.1808 **Identification of Core Components and Transient Interactors of the Peroxisomal Importomer by Dual-Track Stable Isotope Labeling with Amino Acids in Cell Culture Analysis**
Oeljeklaus, S., Reinartz, B.S., Wolf, J., Wiese, S., Tonillo, J., Podwojski, K., Kuhlmann, K., Stephan, C., Meyer, H.E., Schliebs, W., Brocard, C., Erdmann, R and Warscheid, B.
J. Proteome Res., ***11(4)***, *2567-2580 (2012)*

The importomer complex plays an essential role in the biogenesis of peroxisomes by mediating the translocation of matrix proteins across the organellar membrane. A central part of this highly dynamic import machinery is the docking complex consisting of Pex14p, Pex13p, and Pex17p that is linked to the RING finger complex (Pex2p, Pex10p, Pex12p) via Pex8p. To gain detailed knowledge on the molecular players governing peroxisomal matrix protein import and, thus, the integrity and functionality of peroxisomes, we aimed at a most comprehensive investigation of stable and transient interaction partners of Pex14p, the central component of the importomer. To this end, we performed a thorough quantitative proteomics study based on epitope tagging of Pex14p combined with dual-track stable isotope labeling with amino acids in cell culture-mass spectrometry (SILAC-MS) analysis of affinity-purified Pex14p complexes and statistics. The results led to the establishment of the so far most extensive Pex14p interactome, comprising 9 core and further 12 transient components. We confirmed virtually all known Pex14p interaction partners including the core constituents of the importomer as well as Pex5p, Pex11p, Pex15p, and Dyn2p. More importantly, we identified new transient interaction partners (Pex25p, Hrr25p, Esl2p, prohibitin) that provide a valuable resource for future investigations on the functionality, dynamics, and regulation of the peroxisomal importomer.

3.1809 **Ethanol triggers sphingosine 1-phosphate elevation along with neuroapoptosis in the developing mouse brain**
Chakraborty, G., Saito, M., Shah, R., Mao, R-F., Vadasz, C. and Saito, M.
J. Neurochem., ***121(5)***, *806-817 (2012)*

Our previous studies have indicated that *de novo* ceramide synthesis plays a critical role in ethanol-induced apoptotic neurodegeneration in the 7-day-old mouse brain. In this study, we examined whether the formation of sphingosine 1-phosphate (S1P), a ceramide metabolite, is associated with this apoptotic pathway. Analyses of basal levels of S1P-related compounds indicated that S1P, sphingosine, sphingosine kinase 2, and S1P receptor 1 increased significantly during postnatal brain development. In the 7-day-old mouse brain, sphingosine kinase 2 was localized mainly in neurons. Subcellular fractionation studies of the brain homogenates showed that sphingosine kinase 2 was enriched in the plasma membrane and the synaptic membrane/synaptic vesicle fractions, but not in the nuclear and mitochondrial/lysosomal fractions. Ethanol exposure in 7-day-old mice induced sphingosine kinase 2 activation and increased the brain level of S1P transiently 2–4 h after exposure, followed by caspase 3 activation that peaked around 8 h after exposure. Treatment with dimethylsphingosine, an inhibitor of sphingosine kinases, attenuated the ethanol-induced caspase 3 activation and the subsequent neurodegeneration. These results indicate that ethanol activates sphingosine kinase 2, leading to a transient increase in S1P, which may be involved in neuroapoptotic action of ethanol in the developing brain.

3.1810 **CCL11 elicits secretion of RNases from mouse eosinophils and their cell-free granules**
Shamri, R., Melo, R.C.N., Young, K.M., Bivas-Benita, M., Xenakis, J.J., Spencer, L.A. and Weller, P.F.
FASEB J., ***26(5)***, *2084-2093 (2012)*

Rapid secretion of eosinophil-associated RNases (EARs), such as the human eosinophilic cationic protein (ECP), from intracellular granules is central to the role of eosinophils in allergic diseases and host immunity. Our knowledge regarding allergic inflammation has advanced based on mouse experimental models. However, unlike human eosinophils, capacities of mouse eosinophils to secrete granule proteins have been controversial. To study mechanisms of mouse eosinophil secretion and EAR release, we combined an RNase assay of mouse EARs with ultrastructural studies. *In vitro*, mouse eosinophils stimulated with the chemokine eotaxin-1 (CCL11) secreted enzymatically active EARs (EC_{50} 5 nM) by piecemeal degranulation. *In vivo*, in a mouse model of allergic airway inflammation, increased airway eosinophil infiltration (24-fold) correlated with secretion of active RNases (3-fold). Moreover, we found that eosinophilic inflammation in mice can involve eosinophil cytolysis and release of cell-free granules. Cell-free mouse eosinophil granules expressed functional CCR3 receptors and secreted their granule proteins, including EAR and eosinophil peroxidase in response to CCL11. Collectively, these data demonstrate chemokine-dependent secretion of EARs from both intact mouse eosinophils and their cell-free granules, findings pertinent to understanding the pathogenesis of eosinophil-associated diseases, in which EARs are key factors.

3.1811 Expression and subcellular distribution of gephyrin in non-neuronal tissues and cells
Nawrotzki, R., Islinger, M., Vogel, I., Völkl, A. and Kirsch, J.
Histochem. Cell. Biol., ***137(4)***, *471-482 (2012)*

Gephyrin is a scaffolding protein required for the accumulation of inhibitory neurotransmitter receptors at neuronal postsynaptic membranes. In non-neuronal tissues, gephyrin is indispensible for the biosynthesis of molybdenum cofactor, the prosthetic group of oxidoreductases including sulfite oxidase and xanthine oxidase. However, the molecular and cellular basis of gephyrin's non-neuronal function is poorly understood; in particular, the roles of its splice variants remain enigmatic. Here, we used cDNA screening as well as Northern and immunoblot analyses to show that mammalian liver contains only a limited number of gephyrin splice variants, with the C3-containing variant being the predominant isoform. Using new and established anti-gephyrin antibodies in immunofluorescence and subcellular fractionation studies, we report that gephyrin localizes to the cytoplasm of both tissue hepatocytes and cultured immortalized cells. These findings were corroborated by RNA interference studies in which the cytosolic distribution was found to be abolished. Finally, by blue-native PAGE we show that cytoplasmic gephyrin is part of a ~600 kDa protein complex of yet unknown composition. Our data suggest that the expression pattern of non-neuronal gephyrin is simpler than indicated by previous evidence. In addition, gephyrin's presence in a cytosolic 600 kDa protein complex suggests that its metabolic and/or other non-neuronal functions are exerted in the cytoplasm and are not confined to a particular subcellular compartment.

3.1812 Nesca, a novel neuronal adapter protein, links the molecular motor kinesin with the pre-synaptic membrane protein, syntaxin-1, in hippocampal neurons
MacDonald, J.I.S., Dietrich, A., Gamble, S., Hryciw, T., Grant, R.I. and Meakin, S.O.
J. Neurochem., ***121(6)***, *861-880 (2012)*

Vesicular transport in neurons plays a vital role in neuronal function and survival. Nesca is a novel protein that we previously identified and herein describe its pattern of expression, subcellular localization and protein–protein interactions both *in vitro* and *in vivo*. Specifically, a large proportion of Nesca is in tight association with both actin and microtubule cytoskeletal proteins. Nesca binds to F-actin, microtubules, βIII and acetylated α-tubulin, but not neurofilaments or the actin-binding protein drebrin, in *in vitro*-binding assays. Nesca co-immunoprecipitates with kinesin heavy chain (KIF5B) and kinesin light-chain motors as well as with the synaptic membrane precursor protein, syntaxin-1, and is a constituent of the post-synaptic density. Moreover, *in vitro*-binding assays indicate that Nesca directly binds KIF5B, kinesin light-chain and syntaxin-1. In contrast, Nesca does not co-immunoprecipitate with the kinesin motors KIF1B, KIF3A nor does it bind syntaxin-4 or the synaptosome-associated protein 25 kDa (SNAP-25) *in vitro*. Nesca expression in neurons is highly punctuate, co-stains with syntaxin-1, and is found in fractions containing markers of early endosomes and Golgi suggesting that it is involved in vesicular transport. Collectively, these data suggest that Nesca functions as an adapter involved in neuronal vesicular transport including vesicles containing soluble *N*-ethylmaleimide sensitive factor attachment protein receptors that are essential to exocytosis.

3.1813 Proteasomal degradation of the metabotropic glutamate receptor 1α is mediated by Homer-3 via the proteasomal S8 ATPase
Rezvani, K., Baalman, K., Teng, Y., Mee, M.P., Dawson, S.P., Wang, H., De Biasi, M. and Mayer, R.J.

J. Neurochem., ***122(1)***, *24-37 (2012)*

The metabotropic glutamate receptors (mGluRs) fine-tune the efficacy of synaptic transmission. This unique feature makes mGluRs potential targets for the treatment of various CNS disorders. There is ample evidence to show that the ubiquitin proteasome system mediates changes in synaptic strength leading to multiple forms of synaptic plasticity. The present study describes a novel interaction between post-synaptic adaptors, long Homer-3 proteins, and one of the 26S proteasome regulatory subunits, the S8 ATPase, that influences the degradation of the metabotropic glutamate receptor 1α (mGluR1α). We have shown that the two human long Homer-3 proteins specifically interact with human proteasomal S8 ATPase. We identified that mGluR1α and long Homer-3s immunoprecipitate with the 26S proteasome both *in vitro* and *in vivo*. We further found that the mGluR1α receptor can be ubiquitinated and degraded by the 26S proteasome and that Homer-3A facilitates this process. Furthermore, the siRNA mediated silencing of Homer-3 led to increased levels of total and plasma membrane-associated mGluR1α receptors. These results suggest that long Homer-3 proteins control the degradation of mGluR1α receptors by shuttling ubiquitinated mGluR-1α receptors to the 26S proteasome via the S8 ATPase which may modulate synaptic transmission.

3.1814 Rab3D regulates amylase levels, not agonist-induced amylase release, in AR42J cells
Limi, S., Ojakian, G., and Raffaniello, R.
Cell. Mol. Biol. Lett., ***17(2)***, *258-273 (2012)*

Rab3D is a low molecular weight GTP-binding protein that associates with secretory granules in exocrine cells. AR42J cells are derived from rat pancreatic exocrine tumor cells and develop an acinar cell-like phenotype when treated with dexamethasone (Dex). In the present study, we examined the role of Rab3D in Dex-treated AR42J cells. Rab3D expression and localization were analyzed by subcellular fractionation and immunoblotting. The role of Rab3D was examined by overexpressing myc-labeled wild-type-Rab3D and a constitutively active form of Rab3D (Rab3D-Q81L) in AR42J cells. We found that Rab3D is predominantly membrane-associated in AR42J cells and co-localizes with zymogen granules (ZG). Following CCK-8-induced exocytosis, amylase-positive ZGs appeared to move towards the periphery of the cell and co-localization between Rab3D and amylase was less complete when compared to basal conditions. Overexpression of WT, but not mutant Rab3D, resulted in an increase in cellular amylase levels. Overexpression of mutant and WT Rab3D did not affect granule morphology, CCK-8-induced secretion, long-term (48 hr) basal amylase release or granule density. We conclude that Rab3D is not involved in agonist-induced exocytosis in AR42J cells. Instead, Rab3D may regulate amylase content in these cells.

3.1815 Substrate-Immobilized HIV-1 Tat Drives VEGFR2/αvβ3–Integrin Complex Formation and Polarization in Endothelial Cells
Urbinati, C., Ravelli, C., Tanghetti, E., Belleri, M., Giacopuzzi, E., Monti, E., Presta, M. and Rusnati, M.
Arterioscler. Thromb. Vasc. Biol., ***32***, *e25-e34 (2012)*

Objective—The HIV-1 transactivating factor (Tat) possesses features typical of both cell-adhesive and angiogenic growth factor (AGF) proteins, inducing endothelial cell (EC) adhesion and proangiogenic activation. Tat was exploited to investigate the events triggered by EC adhesion to substrate-bound AGF that lead to proangiogenic activation.
Methods and Results—Immobilized Tat induces actin cytoskeleton organization, formation of $\alpha_v\beta_3$ integrin$^+$focal adhesion plaques, and recruitment of vascular endothelial growth factor receptor-2 (VEGFR2) in the ventral plasma membrane of adherent ECs. Also, acceptor photobleaching fluorescence resonance energy transfer demonstrated that VEGFR2/$\alpha_v\beta_3$ coupling occurs at the basal aspect of Tat-adherent ECs. Cell membrane fractionation showed that a limited fraction of $\alpha_v\beta_3$ integrin and VEGFR2 does colocalize in lipid rafts at the basal aspect of Tat-adherent ECs. VEGFR2 undergoes phosphorylation and triggers pp60src/$ERK_{1/2}$ activation. The use of lipid raft disrupting agents and second messenger inhibitors demonstrated that intact lipid rafts and the VEGFR2/pp60src/$ERK_{1/2}$ pathway are both required for cytoskeleton organization and proangiogenic activation of Tat-adherent ECs.
Conclusion—Substrate-immobilized Tat causes VEGFR2/$\alpha_v\beta_3$ complex formation and polarization at the basal aspect of adherent ECs, VEGFR2/pp60src/$ERK_{1/2}$ phosphorylation, cytoskeleton organization, and proangiogenic activation. These results provide novel insights in the AGF/tyrosine kinase receptor/integrin cross-talk.

3.1816 Serum albumin disrupts Cryptococcus neoformans and Bacillus anthracis extracellular vesicles
Wolf, J.M., Rivera, J and Casadevall, A.
Cell. Microbiol., ***14(5)***, *762-773 (2012)*

For both pathogenic fungi and bacteria, extracellular vesicles have been shown to contain many microbial components associated with virulence, suggesting a role in pathogenesis. However, there are many unresolved issues regarding vesicle synthesis and stability, including the fact that vesicular packaging for extracellular factors involved in virulence must also have a mechanism for vesicle unloading. Consequently, we studied the kinetics of vesicle production and stability using [1-^{14}C] palmitic acid metabolic labelling and dynamic light scattering techniques. *Cryptococcus neoformans* vesicles were produced throughout all stages of fungal culture growth and they were stable once isolated. Density gradient analysis revealed that only a portion of the vesicle population carried cryptococcal polysaccharide, implying heterogeneity in vesicular cargo. Vesicle incubation with macrophages resulted in rapid vesicle instability, a phenomenon that was ultimately associated with serum albumin. Additionally, albumin, along with mouse serum and murine immunoglobulin destabilized *Bacillus anthracis* vesicles, but the effect was not observed with ovalbumin or keyhole limpet haemocyanin, demonstrating that this phenomenon is neither host-, microbe- nor protein-specific. Our findings strongly suggest that cryptococcal vesicles are short-lived *in vivo* and vesicle destabilization is mediated by albumin. The ability of albumin to promote vesicular offload through destabilization indicates a new activity for this abundant serum protein.

3.1817 Kaposi's sarcoma-associated herpesvirus interacts with EphrinA2 receptor to amplify signaling essential for productive infection
Chakraborty, S., Valiya, M., Bottero, V. and Chandran, B.
PNAS Plus, ***109***, *E1163-E1172 (2012)*

Kaposi's sarcoma-associated herpesvirus (KSHV), etiologically associated with Kaposi's sarcoma, uses integrins (α3β1, αVβ3, and αVβ5) and associated signaling to enter human dermal microvascular endothelial cells (HMVEC-d), an in vivo target of infection. KSHV infection activated c-Cbl, which induced the selective translocation of KSHV into lipid rafts (LRs) along with the α3β1, αVβ3, and xCT receptors, but not αVβ5. LR-translocated receptors were monoubiquitinated, leading to productive macropinocytic entry, whereas non-LR–associated αVβ5 was polyubiquitinated, leading to clathrin-mediated entry that was targeted to lysosomes. Because the molecule(s) that integrate signal pathways and productive KSHV macropinocytosis were unknown, we immunoprecipitated KSHV-infected LR fractions with anti-α3β1 antibodies and analyzed them by mass spectrometry. The tyrosine kinase EphrinA2 (EphA2), implicated in many cancers, was identified in this analysis. EphA2 was activated by KSHV. EphA2 was also associated with KSHV and integrins (α3β1 and αVβ3) in LRs early during infection. Preincubation of virus with soluble EphA2, knockdown of EphA2 by shRNAs, or pretreatment of cells with anti-EphA2 monoclonal antibodies or tyrosine kinase inhibitor dasatinib significantly reduced KSHV entry and gene expression. EphA2 associates with c-Cbl-myosin IIA and augmented KSHV-induced Src and PI3-K signals in LRs, leading to bleb formation and macropinocytosis of KSHV. EphA2 shRNA ablated macropinocytosis-associated signaling events, virus internalization, and productive nuclear trafficking of KSHV DNA. Taken together, these studies demonstrate that the EphA2 receptor acts as a master assembly regulator of KSHV-induced signal molecules and KSHV entry in endothelial cells and suggest that the EphA2 receptor is an attractive target for controlling KSHV infection.

3.1818 Generation and Nuclear Translocation of Sumoylated Transmembrane Fragment of Cell Adhesion Molecule L1
Lutz, D., Wolters-Eisfeld, G., Joshi, G., Djogo, N., Jakovcevski, I., Schachner, M. and Kleene, R.
J. Biol. Chem., ***287(21)***, *17161-17175 (2012)*

The functions of the cell adhesion molecule L1 in the developing and adult nervous system are triggered by homophilic and heterophilic interactions that stimulate signal transductions that activate cellular responses. Here, we show that stimulation of signaling by function-triggering L1 antibodies or L1-Fc leads to serine protease-dependent cleavage of full-length L1 at the plasma membrane and generation of a sumoylated transmembrane 70-kDa fragment comprising the intracellular and transmembrane domains and part of the extracellular domain. The 70-kDa transmembrane fragment is transported from the plasma membrane to a late endosomal compartment, released from endosomal membranes into the cytoplasm, and transferred from there into the nucleus by a pathway that depends on importin and chromatin-modifying protein 1. Mutation of the sumoylation site at Lys^{1172} or of the nuclear localization signal at Lys^{1147}

abolished L1-stimulated generation or nuclear import of the 70-kDa fragment, respectively. Nuclear import of the 70-kDa fragment may activate cellular responses in parallel or in association with phosphorylation-dependent signaling pathways. Alterations in the levels of the 70-kDa fragment during development and in the adult after spinal cord injury or in a mouse model of Alzheimer disease suggest that this fragment is functionally implicated in development, regeneration, neurodegeneration, tumorigenesis, and possibly synaptic plasticity in the mature nervous system.

3.1819 Phospholipase A2 activating protein is required for 1α,25-dihydroxyvitamin D3 dependent rapid activation of protein kinase C via Pdia3
Doroudi, M., Schwartz, Z. and Boyan, B.D.
J. Steroid Biochem. Mol. Biol., ***132***, *48-56 (2012)*

1 α ,25-Dihydroxyvitamin D_3 (1,25D3) regulates musculoskeletal cells via two different mechanisms: vitamin D receptor (VDR)-dependent gene transcription and rapid membrane-signaling via VDR as well as protein disulfide isomerase, family A, member 3 (Pdia3). In chondrocytes from the costochondral cartilage growth zone (GC), ligand binding to Pdia3 causes a rapid increase in phospholipase A_2 (PLA_2) activity leading to release of arachidonic acid and formation of lysophospholipid (LPL). LPL activates phospholipase C (PLC), and resulting inositol trisphosphate (IP_3) and diacylglycerol contribute to PKC α activation and downstream activation of ERK1/2. PLA_2 activating protein (PLAA) is increased in the growth zone of rat growth plates suggesting that it mediates the 1,25D3-dependent pathway. This study examined the role of PLAA in mediating 1,25D3-dependent PKC activation using GC cells and MC3T3-E1 wild-type and PLAA-silenced osteoblasts as models. PLAA, Pdia3, and caveolin-1 (Cav-1) were detected in plasma membranes and caveolae of GC and MC3T3-E1 cells. Pdia3-immunoprecipitated samples were positive for PLAA only after 1,25D3 treatment. Cav-1 was detected when immunoprecipitated with anti-Pdia3 and anti-PLAA in both vehicle and 1,25D3 treated cells. These observations were confirmed by immunohistochemistry. 1,25D3 failed to activate PLA_2 and PKC or cause PGE_2 release in PLAA-silenced cells. PLAA-antibody successfully blocked the PLAA protein and consequently suppressed PKC activity in GC and MC3T3-E1 cells. Crosslinking studies confirmed the localization of PLAA on the extracellular face on the plasma membrane in untreated MC3T3-E1 cells. Taken together, our results suggest that PLAA is an important mediator of 1 α ,25$(OH)_2D_3$ rapid membrane mediated signaling. 1 α ,25$(OH)_2D_3$ likely causes conformational changes bringing Pdia3 into proximity with PLAA, and aiding in transducing the signal from caveolae to the plasma membrane.

3.1820 Polyunsaturated fatty acid supplements modulate mast cell membrane microdomain composition
Basiouni, S., Stöckel, K., Fuhrmann, H. and Schumann, J.
Cellular Immunol., ***275***, *42-46 (2012)*

In the present study, the lipid raft composition of a canine mastocytoma cell line (C2) was analyzed. Lipid rafts were well separated from non-raft plasma membranes using a detergent-free isolation technique. To study the influence of n-3 and n-6 polyunsaturated fatty acids (PUFA) on raft fatty acid composition in comparison to non-raft cell membrane, C2 were supplemented with one of the following: α -linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, linoleic acid or arachidonic acid. Enrichment of the culture medium with a specific PUFA resulted in an increase in the content of this fatty acid both in rafts and non-raft membranes. Contents of cholesterol and protein were found not to be affected by the changes in the fatty acid profiles. In conclusion, our data provide strong evidence that PUFA modulate lipid composition and physiological properties of membrane micro domains of mast cells which in turn may have effects on mast cell function.

3.1821 Proteomic Analysis of Rta2p-Dependent Raft-Association of Detergent-Resistant Membranes in Candida albicans
Wang, L., Jia, Y., Tang, R-J., Xu, Z., Cao, Y-B., Jia, X-M. and Jiang, Y-Y.
Plos One, ***7(5)***, *e37768 (2012)*

In *Candida albicans*, lipid rafts (also called detergent-resistant membranes, DRMs) are involved in many cellular processes and contain many important proteins. In our previous study, we demonstrated that Rta2p was required for calcineurin-mediated azole resistance and sphingoid long-chain base release in *C. albicans*. Here, we found that Rta2p was co-localized with raft-constituted ergosterol on the plasma membrane of *C. albicans*. Furthermore, this membrane expression pattern was totally disturbed by inhibitors of either ergosterol or sphingolipid synthesis. Biochemical fractionation of DRMs together with immunoblot uncovered that Rta2p, along with well-known DRM-associated proteins (Pma1p and Gas1p

homologue), was associated with DRMs and their associations were blocked by inhibitors of either ergosterol or sphingolipid synthesis. Finally, we used the proteomic analysis together with immunoblot and identified that Rta2p was required for the association of 10 proteins with DRMs. These 5 proteins (Pma1p, Gas1p homologue, Erg11p, Pmt2p and Ali1p) have been reported to be DRM-associated and also that Erg11p is a well-known target of azoles in *C. albicans*. In conclusion, our results showed that Rta2p was predominantly localized in lipid rafts and was required for the association of certain membrane proteins with lipid rafts in *C. albicans*.

3.1822 Identification of a Wnt-induced protein complex by affinity proteomics using an antibody that recognizes a sub-population of β-catenin

Layton, M.J., Faux, M.C., Church, N:L., Catimel, B., Kershaw, N:J., Kapp, E.A., Nowell, C., Coates, J.L., Burgess, A.W. and Simpson, R.J.

*Biochim. Biophys. Acta, **1824**, 925-937 (2012)*

β-catenin is a signaling protein with diverse functions in cell adhesion and Wnt signaling. Although β-catenin has been shown to participate in many protein-protein interactions, it is not clear which combinations of β-catenin-interacting proteins form discrete complexes. We have generated a novel antibody, termed 4B3, which recognizes only a small subset of total cellular β-catenin. Affinity proteomics using 4B3, in combination with subcellular fractionation, has allowed us to define a discrete trimeric complex of β-catenin, α-catenin and the tumor suppressor APC, which forms in the cytoplasm in response to Wnt signaling. Depletion of the limiting component of this complex, APC, implicates the complex in mediating Wnt-induced changes in cell-cell adhesion. APC is also essential for N-terminal phosphorylation of β-catenin within this complex. Each component of β-catenin/APC/α-catenin complex co-exists in other protein complexes, thus use of a selective antibody for affinity proteomics has allowed us to go beyond the generation of a list of potential β-catenin-interacting proteins, and define when and where a specific complex forms.

3.1823 Impaired neurotransmission in ether lipid-deficient nerve terminals

Brodde, A., Teigler, A., Brugger, B., Lehmann, W.D., Wieland, F., Berger, J. and Just, W.W.

*Hum. Mol. Genet., **21(12)**, 2713-2724 (2012)*

Isolated defects of ether lipid (EL) biosynthesis in humans cause rhizomelic chondrodysplasia punctata type 2 and type 3, serious peroxisomal disorders. Using a previously described mouse model [Rodemer, C., Thai, T.P., Brugger, B., Kaercher, T., Werner, H., Nave, K.A., Wieland, F., Gorgas, K., and Just, W.W. (2003) Inactivation of ether lipid biosynthesis causes male infertility, defects in eye development and optic nerve hypoplasia in mice. *Hum. Mol. Genet.*, **12**, 1881–1895], we investigated the effect of EL deficiency in isolated murine nerve terminals (synaptosomes) on the pre-synaptic release of the neurotransmitters (NTs) glutamate and acetylcholine. Both Ca^{2+}-dependent exocytosis and Ca^{2+}-independent efflux of the transmitters were affected. EL-deficient synaptosomes respire at a reduced rate and exhibit a lowered adenosin-5′-triphosphate/adenosine diphosphate (ATP/ADP) ratio. Consequently, ATP-driven processes, such as synaptic vesicle cycling and maintenance of Na^+, K^+ and Ca^{2+} homeostasis, might be disturbed. Analyzing reactive oxygen species in EL-deficient neural and non-neural tissues revealed that plasmalogens (PLs), the most abundant EL species in mammalian central nervous system, considerably contribute to the generation of the lipid peroxidation product malondialdehyde. Although EL-deficient tissue contains less lipid peroxidation products, fibroblasts lacking ELs are more susceptible to induced oxidative stress. In summary, these results suggest that due to the reduced energy state of EL-deficient tissue, the Ca^{2+}-independent efflux of NTs increases while the Ca^{2+}-dependent release declines. Furthermore, lack of PLs is mainly compensated for by an increase in the concentration of phosphatidylethanolamine and results in a significantly lowered level of lipid peroxidation products in the brain cortex and cerebellum.

3.1824 Mutagenesis of the DI/DIII Linker in Dengue Virus Envelope Protein Impairs Viral Particle Assembly

De Wispelaere, M. and Yang, P.L.

*J. Virol., **86(13)**, 7072-7083 (2012)*

The dengue virus (DV) envelope (E) protein is important in mediating viral entry and assembly of progeny virus during cellular infection. Domains I and III (DI and DIII, respectively) of the DV E protein are connected by a highly conserved but poorly ordered region, the DI/DIII linker. Although the flexibility of

the DI/DIII linker is thought to be important for accommodating the structural rearrangements undergone by the E protein during viral entry, the function of the linker in the DV infectious cycle is not well understood. In this study, we performed site-directed mutagenesis on conserved residues in the DI/DIII linker of the DV2 E protein and showed that the resulting mutations had little or no effect on the entry process but greatly affected virus assembly. Biochemical fractionation and immunofluorescence microscopy experiments performed on infectious virus as well as in a virus-like particle (VLP) system indicate that the DI/DIII linker mutants express the DV structural proteins at the sites of particle assembly near the ER but fail to form infectious particles. This defect is not due to disruption of E's interaction with prM and pr in immature and mature virions, respectively. Serial passaging of the DV2 mutant E-Y299F led to the identification of a mutation in the membrane-proximal stem region of E that fully compensates for the assembly defect of this DI/DIII linker mutant. Together, our results suggest a critical and previously unidentified role for the E protein DI/DIII linker region during the DV2 assembly process.

3.1825 Human Herpesvirus 8 Interferon Regulatory Factor-Mediated BH3-Only Protein Inhibition via Bid BH3-B Mimicry

Choi, Y.B., Sandford, G. and Nicholas, J.
*PloS Pathogens, **8(6)**, e1002748 (2012)*

Viral replication efficiency is in large part governed by the ability of viruses to counteract pro-apoptotic signals induced by infection of host cells. For HHV-8, viral interferon regulatory factor-1 (vIRF-1) contributes to this process in part via inhibitory interactions with BH3-only protein (BOP) Bim, recently identified as an interaction partner of vIRF-1. Here we recognize that the Bim-binding domain (BBD) of vIRF-1 resembles a region (BH3-B) of Bid, another BOP, which interacts intramolecularly with the functional BH3 domain of Bid to inhibit it pro-apoptotic activity. Indeed, vIRF-1 was found to target Bid in addition to Bim and to interact, via its BBD region, with the BH3 domain of each. In functional assays, BBD could substitute for BH3-B in the context of Bid, to suppress Bid-induced apoptosis in a BH3-binding-dependent manner, and vIRF-1 was able to protect transfected cells from apoptosis induced by Bid. While vIRF-1 can mediate nuclear sequestration of Bim, this was not the case for Bid, and inhibition of Bid and Bim by vIRF-1 could occur independently of nuclear localization of the viral protein. Consistent with this finding, direct BBD-dependent inactivation by vIRF-1 of Bid-induced mitochondrial permeabilization was demonstrable *in vitro* and isolated BBD sequences were also active in this assay. In addition to Bim and Bid BH3 domains, BH3s of BOPs Bik, Bmf, Hrk, and Noxa also were found to bind BBD, while those of both pro- and anti-apoptotic multi-BH domain Bcl-2 proteins were not. Finally, the significance of Bid to virus replication was demonstrated via Bid-depletion in HHV-8 infected cells, which enhanced virus production. Together, our data demonstrate and characterize BH3 targeting and associated inhibition of BOP pro-apoptotic activity by vIRF-1 via Bid BH3-B mimicry, identifying a novel mechanism of viral evasion from host cell defenses.

3.1826 Regulation in the targeting of TRAIL receptor 1 to cell surface via GODZ for TRAIL sensitivity in tumor cells

Oh, Y., Jeon, Y-J., Hong, G-S., Kim, I., Woo, H-N. and Jung, Y-K**.**
*Cell Death and Differentiation, **19(7)**, 1196-1207 (2012)*

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), promote the selective clearing of various malignancies by inducing apoptosis, holding the promise as a potent therapeutic agent for anticancer. Though DR4 and DR5 have high sequence similarity, differential regulation of both receptors in human tumor cells remains largely unexplored. Here, we repot that golgi-specific Asp-His-His-Cys (DHHC) zinc finger protein (GODZ) regulates TRAIL/DR4-mediated apoptosis. Using the SOS protein recruitment–yeast two-hybrid screening, we isolated GODZ that interacted with the death domain of DR4. GODZ binds to DR4, but not to DR5, through the DHHC and the C-terminal transmembrane domain. Expression level of GODZ affects apoptosis of tumor cells triggered by TRAIL, but not that induced by TNF-α/cycloheximide (CHX) or DNA-damaging drugs. In parallel, GODZ functions to localize DR4 to the plasma membrane (PM) via DHHC motif. Also, introduction of mutation into the cysteine-rich motif of DR4 results in its mistargeting and attenuates TRAIL- or GODZ-mediated apoptosis. Interestingly, GODZ expression is highly downregulated in Hep-3B tumor cells, which show resistance to TRAIL. However, reconstitution of GODZ expression enhances the targeting of DR4 to cell surface and sensitizes Hep-3B cells to TRAIL. Taken together, these data establish that GODZ is a novel DR4-selective regulator responsible for targeting of DR4 to the PM, and thereby for TRAIL-induced apoptosis.

3.1827 Loss of P-type ATPase ATP13A2/PARK9 function induces general lysosomal deficiency and leads to Parkinson disease neurodegeneration

Dehay, B., Ramirez, A., Martinez-Vicente, M., Perier, C., Canron, M-H., Doudnikoff, E., Vital, A., Vila, M., Klein, C. and Bezard, E.

*PNAS, **109(24)**, 9611-9616 (2011)*

Parkinson disease (PD) is a progressive neurodegenerative disorder pathologically characterized by the loss of dopaminergic neurons from the substantia nigra pars compacta and the presence, in affected brain regions, of protein inclusions named Lewy bodies (LBs). The *ATP13A2* gene (locus PARK9) encodes the protein ATP13A2, a lysosomal type 5 P-type ATPase that is linked to autosomal recessive familial parkinsonism. The physiological function of ATP13A2, and hence its role in PD, remains to be elucidated. Here, we show that PD-linked mutations in ATP13A2 lead to several lysosomal alterations in ATP13A2 PD patient-derived fibroblasts, including impaired lysosomal acidification, decreased proteolytic processing of lysosomal enzymes, reduced degradation of lysosomal substrates, and diminished lysosomal-mediated clearance of autophagosomes. Similar alterations are observed in stable ATP13A2-knockdown dopaminergic cell lines, which are associated with cell death. Restoration of ATP13A2 levels in ATP13A2-mutant/depleted cells restores lysosomal function and attenuates cell death. Relevant to PD, ATP13A2 levels are decreased in dopaminergic nigral neurons from patients with PD, in which ATP13A2 mostly accumulates within Lewy bodies. Our results unravel an instrumental role of ATP13A2 deficiency on lysosomal function and cell viability and demonstrate the feasibility and therapeutic potential of modulating ATP13A2 levels in the context of PD.

3.1828 Mo1816 The NHE3 Interacting PDZ Protein NHERF2 is Strongly Lipid Raft-Associated and Determines the Raft Association of the Apical Na+/H+ Exchanger NHE3 in Murine Small Intestinal Brush Border Membrane

Sultan, A., Riederer, B., Xia, W., Lamprecht, G., Lissner, S., Yun, C., de JOnge, H., Gessner, J.E., Donowitz, M. and Seidler, U.

*Gastroenterology, **142(5)**, Suppl. 1, S691-S692 (2012)*

Abstract not available

3.1829 The GTPase ARFRP1 controls the lipidation of chylomicrons in the Golgi of the intestinal epithelium

Jaschke, A., Chung, B., Hesse, D., Kluge, R., Zahn, C., Moser, M., Petzke, K-J., Brigelius-Flohe, R., Puchkov, D., Koepsell, H., Heeren, J., Joost, H-G., and Schürmann, A.

*Hum. Mol. Genet., **21(14)**, 3128-3142 (2012)*

The uptake and processing of dietary lipids by the small intestine is a multistep process that involves several steps including vesicular and protein transport. The GTPase ADP-ribosylation factor-related protein 1 (ARFRP1) controls the ARF-like 1 (ARL1)-mediated Golgi recruitment of GRIP domain proteins which in turn bind several Rab-GTPases. Here, we describe the essential role of ARFRP1 and its interaction with Rab2 in the assembly and lipidation of chylomicrons in the intestinal epithelium. Mice lacking Arfrp1 specifically in the intestine ($Arfrp1^{vil-/-}$) exhibit an early post-natal growth retardation with reduced plasma triacylglycerol and free fatty acid concentrations. $Arfrp1^{vil-/-}$ enterocytes as well as Arfrp1 mRNA depleted Caco-2 cells absorbed fatty acids normally but secreted chylomicrons with a markedly reduced triacylglycerol content. In addition, the release of apolipoprotein A-I (ApoA-I) was dramatically decreased, and ApoA-I accumulated in the $Arfrp1^{vil-/-}$ epithelium, where it predominantly co-localized with Rab2. The release of chylomicrons from Caco-2 was markedly reduced after the suppression of Rab2, ARL1 and Golgin-245. Thus, the GTPase ARFRP1 and its downstream proteins are required for the lipidation of chylomicrons and the assembly of ApoA-I to these particles in the Golgi of intestinal epithelial cells.

3.1830 The Amyloid Precursor Protein Copper Binding Domain Histidine Residues 149 and 151 Mediate APP Stability and Metabolism

Spoerri, L., Vella, L.J., Pham, C.L.L., Barnham, K.J. and Cappai, R.

*J. Biol. Chem., **287(32)**, 26840-26853 (2012)*

One of the key pathological hallmarks of Alzheimer disease (AD) is the accumulation of the APP-derived amyloid β peptide (Aβ) in the brain. Altered copper homeostasis has also been reported in AD patients and is thought to increase oxidative stress and to contribute to toxic Aβ accumulation and regulate APP

metabolism. The potential involvement of the N-terminal APP copper binding domain (CuBD) in these events has not been investigated. Based on the tertiary structure of the APP CuBD, we examined the histidine residues of the copper binding site (His^{147}, His^{149}, and His^{151}). We report that histidines 149 and 151 are crucial for CuBD stability and APP metabolism. Co-mutation of the APP CuBD His^{149} and His^{151} to asparagine decreased APP proteolytic processing, impaired APP endoplasmic reticulum-to-Golgi trafficking, and promoted aberrant APP oligomerization in HEK293 cells. Expression of the triple H147N/H149N/H151N-APP mutant led to up-regulation of the unfolded protein response. Using recombinant protein encompassing the APP CuBD, we found that insertion of asparagines at positions 149 and 151 altered the secondary structure of the domain. This study identifies two APP CuBD residues that are crucial for APP metabolism and suggests an additional role of this domain in APP folding and stability besides its previously identified copper binding activity. These findings are of major significance for the design of novel AD therapeutic drugs targeting this APP domain.

3.1831 Nascent high density lipoproteins formed by ABCA1 resemble lipid rafts and are structurally organized by three apoA-I monomers
Sorci-Thomas, M.G., Owen, J.S., Fulp, B., Bhat, S., Zhu, X., Parks, J.S., Shah, D., Jerome, W.G., Gerelus, M., Zabalawi, M. and Thomas, M.J.
J. Lipid Res., ***53***, *1890-1909 (2012)*

This report details the lipid composition of nascent HDL (nHDL) particles formed by the action of the ATP binding cassette transporter A1 (ABCA1) on apolipoprotein A-I (apoA-I). nHDL particles of different size (average diameters of ~12, 10, 7.5, and <6 nm) and composition were purified by size-exclusion chromatography. Electron microscopy suggested that the nHDL were mostly spheroidal. The proportions of the principal nHDL lipids, free cholesterol, glycerophosphocholine, and sphingomyelin were similar to that of lipid rafts, suggesting that the lipid originated from a raft-like region of the cell. Smaller amounts of glucosylceramides, cholesteryl esters, and other glycerophospholipid classes were also present. The largest particles, ~12 nm and 10 nm diameter, contained ~43% free cholesterol, 2–3% cholesteryl ester, and three apoA-I molecules. Using chemical cross-linking chemistry combined with mass spectrometry, we found that three molecules of apoA-I in the ~9–14 nm nHDL adopted a belt-like conformation. The smaller (7.5 nm diameter) spheroidal nHDL particles carried 30% free cholesterol and two molecules of apoA-I in a twisted, antiparallel, double-belt conformation. Overall, these new data offer fresh insights into the biogenesis and structural constraints involved in forming nascent HDL from ABCA1.

3.1832 The major isoforms of Bim contribute to distinct biological activities that govern the processes of autophagy and apoptosis in interleukin-7 dependent lymphocytes
Ruppert, S.M., Li, W., Zhang, G., Carlson, A.L., Limaye, A., Durum, S.K. and Khaled, A.R.
Biochim. Biophys. Acta, ***1823****, 1877-1893 (2012)*

Bim is a BH3-only member of the Bcl-2 family that enables the death of T-cells. Partial rescue of cytokine-deprived T-cells occurs when Bim and the receptor for the T-cell growth factor, interleukin-7, are deleted, implicating Bim as a possible target of interleukin-7-mediated signaling. Alternative splicing yields three major isoforms: BimEL, BimL and BimS. To study the effect of Bim deficiency and define the function of the major isoforms, Bim-containing and Bim-deficient T-cells, dependent on interleukin-7 for growth, were used. Loss of total Bim in interleukin-7-deprived T-cells resulted in delayed apoptosis. However, loss of Bim also impeded the later degradative phase of autophagy. p62, an autophagy-adaptor protein which is normally degraded, accumulated in Bim deficient cells. To explain this, BimL was found to support acidification of lysosomes that later may associate with autophagic vesicles. Key findings showed that inhibition of lysosomal acidification accelerated death upon interleukin-7 withdrawal only in Bim-containing T-cells. intereukin-7 dependent T-cells lacking Bim were less sensitive to inhibition of lysosomal acidification. BimL co-immunoprecipitated with dynein and Lamp1-containing vesicles, indicating BimL could be an adaptor for dynein to facilitate loading of lysosomes. In Bim deficient T-cells, lysosome-tracking probes revealed vesicles of less acidic pH. Over-expression of BimL restored acidic vesicles in Bim deficient T-cells, while other isoforms, BimEL and BimS, promoted intrinsic cell death. These results reveal a novel role for BimL in lysosomal positioning that may be required for the formation of degradative autolysosomes.

3.1833 Melittin initiates dopamine transporter internalization and recycling in transfected HEK-293 cells
Keith, D.J., Wolfrum, K., Eshleman, A.J. and Janowsky, A.

Eur. J. Pharmacol., ***690****, 13-21 (2012)*

The dopamine transporter removes the neurotransmitter from the synapse, regulating dopamine availability. The transporter can be internalized and its function is blocked by cocaine and other ligands. Melittin inhibits dopamine transporter function and causes internalization of the recombinant transporter in stably transfected HEK-293 cells, but the specific pathways for internalization and disposition of the transporter are unknown. Here we report that melittin treatment increased both transporter internalization and colocalization with clathrin, effects that were blocked by pretreatment with cocaine. Density gradient centrifugation revealed that melittin treatment caused the dopamine transporter to associate with a density fraction containing the early endosome marker Rab 5A. Confocal microscopy revealed that melittin treatment also increased transporter colocalization with Rab 5A and decreased colocalization with the late endosome marker Rab 7 and the recycling endosome marker Rab 11. Following 60 min of melittin treatment, the transporter was trafficked back to the membrane. By comparison, phorbol ester treatment increased transporter colocalization with early endosome antigen 1 and Rab 7 in a time-dependent manner. Cocaine treatment alone does not affect transporter trafficking in these cells. Results indicate multiple dopamine transporter internalization and recycling pathways that depend on transporter-ligand interactions and post-translational modifications.

3.1834 Elucidating the pre- and post-nuclear intracellular processing of 1,4-dihydropyridine based gene delivery carriers

Hyvönen, Z., Hämäläinen, V., Ruponen, M., Lucas, B., Rejman, J., Vercauteren, D., Demeester, J., De Smedt, S. and Braeckmans, K.
J. Controlled Release, ***162****, 167-175 (2012)*

The low transfection efficacy of non-viral gene delivery systems limits the therapeutic application of these vectors. Besides the inefficient release of the complexes or pDNA from endolysosomes into the cytoplasm or poor nuclear uptake, the nuclear and post-nuclear processing might unfavorably affect the transgene expression. Positively charged amphiphilic 1,4-dihydropyridine (1,4-DHP) derivatives were earlier proposed as a promising tool for the delivery of DNA into target cells *in vitro* and *in vivo*. However, the structure/activity relationship of these carriers is poorly understood as yet. In this work we studied the intracellular processing of complexes, composed of three structurally related 1,4-DHP derivatives, in a retinal pigment epithelial (ARPE-19) cell line. The pre- and post-nuclear processing of the complexes was quantified on the nuclear, mRNA and transgene expression level. Here we show that the interaction of 1,4-DHP complexes with the cell membrane temporarily increases the permeability of the ARPE-19 cell membrane for small molecular compounds. However, the main mechanism for internalization of 1,4-DHP complexes is endocytosis. We found that all examined derivatives are able to destabilize endosomal membranes by lipid exchange upon acidification. In addition, the buffering capacity of some of the compounds may contribute to the endosomal escape of the complexes as well through the proton sponge effect. Previously we reported that cellular uptake of 1,4-DHP complexes does not correlate with transgene expression. In this study we surprisingly revealed that there is no correlation between the amount of plasmids taken up by the cell and the amount of plasmids found in the cell nucleus. Furthermore, it was found that a high amount of plasmid in the nucleus does not ensure high mRNA expression, likely due to remaining interactions of the carrier with the plasmids. Neither did the expression of mRNA always result in the production of a functional protein, possibly due to the interaction of free carrier with intracellular components which are involved in the post-translational modification of protein and folding process. Overall, our data suggest that succeeding of both the pre- and the post-nuclear intracellular processes is equally essential for successful transgene expression.

3.1835 N-linked glycosylation of proline-rich membrane anchor (PRiMA) is not required for assembly and trafficking of globular tetrameric acetylcholinesterase

Chan, W.K.B., Chen, V.P., Luk, W.K.W., Choi, R.C.Y. and Tsim, K.W.K.
Neuroscience Lett., ***523****, 71-75 (2012)*

Acetylcholinesterase (AChE) is organized into globular tetramers (G_4) by a structural protein called proline-rich membrane anchor (PRiMA), anchoring it into the cell membrane of neurons in the brain. The assembly of AChE tetramers with PRiMA requires the presence of a C-terminal "t-peptide" in the AChE catalytic subunit ($AChE_T$). The glycosylation of $AChE_T$ is known to be required for its proper assembly and trafficking; however, the role of PRiMA glycosylation in the oligomer assembly has not been revealed. PRiMA is a glycoprotein containing two putative N-linked glycosylation sites. By using site-directed mutagenesis, the asparagine-43 was identified to be the N-linked glycosylation site of PRiMA.

Abolishing glycosylation on mouse PRiMA appeared not to affect its assembly with $AChE_T$, the enzymatic properties of AChE, and the membrane trafficking of PRiMA-linked AChE tetramers. This result is contrary to the reports that glycosylation is essential for conformation and trafficking of membrane glycoproteins.

3.1836 Src kinase-mediates androgen receptor-dependent non-genomic activation of signaling cascade leading to endothelial nitric oxide synthase
Yu, J., Akishita, M., Eto, M., Koizumi, H., Hashimoto, R., Ogawa, S., Tanaka, K., Ouchi, Y. and Okabe, T.
Biochem. Biophys. Res. Comm., ***424***, *538-543 (2012)*

Our previous study has demonstrated that testosterone rapidly activates endothelial nitric oxide synthase (eNOS), enhancing nitric oxide (NO) release from endothelial cells (ECs) via the phosphatidylinositol 3-kinase/Akt (PI3-kinase/Akt) pathway. The upstream regulators of this pathway are unknown. In this study, we further investigated the non-genomic action of testosterone in human aortic ECs. Acute (30 min) activation of eNOS caused by testosterone was unaffected by pretreatment with a transcriptional inhibitor, actinomycin D. Non-permeable testosterone-BSA rapidly induced Akt and eNOS phosphorylation. In contrast, luciferase reporter assay showed that the transcriptional activity of the androgen-responsive element (ARE) was increased by testosterone, but not by testosterone-BSA at 2 h after stimulation. Immunostaining displayed co-localization of androgen receptor (AR) with caveolin-1. Fractional analysis showed that AR was expressed in caveolae-enriched membrane fractions. Immunoprecipitation assays revealed the association of AR with caveolin-1 and c-Src, suggesting complex formation among them. Testosterone rapidly increased the phosphorylation of c-Src on Tyr416, which was inhibited by an AR antagonist and by siRNA for AR. PP2, a specific-inhibitor of Src kinase, abolished the testosterone-induced phosphorylation of Akt and eNOS. Our data indicate that testosterone induces rapid assembly of a membrane signaling complex among AR, caveolin-1 and c-Src, which then facilitates activation of the c-Src/ PI3-kinase/Akt cascade, resulting in activation of eNOS.

3.1837 Pseudo half-molecules of the ABC transporter, COMATOSE, bind Pex19 and target to peroxisomes independently but are both required for activity
Nyathi, Y., Zhang, X., Baldwin, J.M., Bernhardt, K., Johnson, B., Baldwin, S.A., Theodoulou, F.L. and Baker, A.
FEBS Lett., ***586***, *2280-2286 (2012)*

Peroxisomal ABC transporters of animals and fungi are "half-size" proteins which dimerise to form a functional transporter. However, peroxisomal ABC transporters of land plants are synthesised as a single polypeptide which represents a fused heterodimer. The N- and C-terminal pseudo-halves of COMATOSE (CTS; AtABCD1) were expressed as separate polypeptides which bound Pex19 in vitro and targeted independently to the peroxisome membrane in yeast, where they were stable but not functional. When co-expressed, the pseudo-halves were fully functional as indicated by ATPase activity and rescue of the *pxa1pxa2 Δ* mutant for growth on oleate. The functional significance of heterodimer asymmetry is discussed.

3.1838 Atg9 vesicles are an important membrane source during early steps of autophagosome formation
Yamamoto, H., Kakuta, S., Watanabe, T.M., Kitamura, A., Sekito, T., Kondo-Kakuta, C., Ichikawa, R., Kinjo, M. and Ohsumi, Y.
J. Cell Biol., ***198(2)***, *219-233 (2012)*

During the process of autophagy, cytoplasmic materials are sequestered by double-membrane structures, the autophagosomes, and then transported to a lytic compartment to be degraded. One of the most fundamental questions about autophagy involves the origin of the autophagosomal membranes. In this study, we focus on the intracellular dynamics of Atg9, a multispanning membrane protein essential for autophagosome formation in yeast. We found that the vast majority of Atg9 existed on cytoplasmic mobile vesicles (designated Atg9 vesicles) that were derived from the Golgi apparatus in a process involving Atg23 and Atg27. We also found that only a few Atg9 vesicles were required for a single round of autophagosome formation. During starvation, several Atg9 vesicles assembled individually into the preautophagosomal structure, and eventually, they are incorporated into the autophagosomal outer membrane. Our findings provide conclusive linkage between the cytoplasmic Atg9 vesicles and autophagosomal membranes and offer new insight into the requirement for Atg9 vesicles at the early step of autophagosome formation.

3.1839 Sphingolipids Regulate the Yeast High-Osmolarity Glycerol Response Pathway
Tanigawa, M., Kihara, A., Terashima, M., Takahara, T. and Maeda, T.
*Mol. Cell. Biol., **32(14)**, 2861-2870 (2012)*

The yeast high-osmolarity glycerol response (HOG) mitogen-activated protein (MAP) kinase pathway is activated in response to hyperosmotic stress via two independent osmosensing branches, the Sln1 branch and the Sho1 branch. While the mechanism by which the osmosensing machinery activates the downstream MAP kinase cascade has been well studied, the mechanism by which the machinery senses and responds to hyperosmotic stress remains to be clarified. Here we report that inhibition of the *de novo* sphingolipid synthesis pathway results in activation of the HOG pathway via both branches. Inhibition of ergosterol biosynthesis also induces activation of the HOG pathway. Sphingolipids and sterols are known to be tightly packed together in cell membranes to form partitioned domains called rafts. Raft-enriched detergent-resistant membranes (DRMs) contain both Sln1 and Sho1, and sphingolipid depletion and hyperosmotic stress have similar effects on the osmosensing machinery of the HOG pathway: dissociation of an Sln1-containing protein complex and elevated association of Sho1 with DRMs. These observations reveal the sphingolipid-mediated regulation of the osmosensing machinery of the HOG pathway.

3.1840 Disruption of Nongenomic Testosterone Signaling in a Model of Spinal and Bulbar Muscular Atrophy
Schindler, M., Fabre, C., de Weille, J., Carreau, S., Mersel, M. and Bakalara, N.
*Mol. Endocrinol., **26(7)**, 1102-1116 (2012)*

As one of the nine hereditary neurodegenerative polyQ disorders, spinal and bulbar muscular atrophy (SBMA) results from a polyQ tract expansion in androgen receptor (AR). Although protein aggregates are the pathological hallmark of many neurodegenerative diseases, their direct role in the neurodegeneration is more and more questioned. To determine the early molecular mechanisms causing motor neuron degeneration in SBMA, we established an *in vitro* system based on the tetracycline-inducible expression of normal (AR20Q), the mutated, 51 glutamine-extended (AR51Q), or polyQ-deleted (AR0Q) AR in NSC34, a motor neuron-like cell line lacking endogenous AR. Although no intracellular aggregates were formed, the expression of the AR51Q leads to a loss of function characterized by reduced neurite outgrowth and to a toxic gain of function resulting in decreased cell viability. In this study, we show that both AR20Q and AR51Q are recruited to lipid rafts in response to testosterone stimulation. However, whereas testosterone induces the activation of the c-jun N-terminal kinase/c-jun pathway via membrane-associated AR20Q, it does not so in NSC34 expressing AR51Q. Phosphorylation of c-jun N-terminal kinase plays a crucial role in AR20Q-dependent survival and differentiation of NSC34. Moreover, c-jun protein levels decrease more slowly in AR20Q- than in AR51Q-expressing NSC34 cells. This is due to a rapid and transient inhibition of glycogen synthase kinase 3α occurring in a phosphatidylinositol 3-kinase-independent manner. Our results demonstrate that the deregulation of nongenomic AR signaling may be involved in SBMA establishment, opening new therapeutic perspectives.

3.1841 The N-Terminal, Polybasic Region of PrPC Dictates the Efficiency of Prion Propagation by Binding to PrPSc
Turnbaugh, J.A., Unterberger, U., Saa, P., Massignan, T., Fluharty, B.R., Bowman, F.P., Miller, M.B., Supattapone, S., Biasini, E. and Harris, D.A.
*J. Neurosci., **32(26)**, 8817-8830 (2012)*

Prion propagation involves a templating reaction in which the infectious form of the prion protein (PrP^{Sc}) binds to the cellular form (PrP^{C}), generating additional molecules of PrP^{Sc}. While several regions of the PrP^{C} molecule have been suggested to play a role in PrP^{Sc} formation based on *in vitro* studies, the contribution of these regions *in vivo* is unclear. Here, we report that mice expressing PrP deleted for a short, polybasic region at the N terminus (residues 23–31) display a dramatically reduced susceptibility to prion infection and accumulate greatly reduced levels of PrP^{Sc}. These results, in combination with biochemical data, demonstrate that residues 23–31 represent a critical site on PrP^{C} that binds to PrP^{Sc} and is essential for efficient prion propagation. It may be possible to specifically target this region for treatment of prion diseases as well as other neurodegenerative disorders due to β-sheet-rich oligomers that bind to PrP^{C}.

3.1842 A Cytotoxic Type III Secretion Effector of Vibrio parahaemolyticus Targets Vacuolar H+-ATPase Subunit c and Ruptures Host Cell Lysosomes
Matsuda, S., Okada, N., Kodama, T., Honda, T. and Iida, T.
*PloS Pathogens, **8(7)**, e1002803 (2012)*

Vibrio parahaemolyticus is one of the human pathogenic vibrios. During the infection of mammalian cells, this pathogen exhibits cytotoxicity that is dependent on its type III secretion system (T3SS1). VepA, an effector protein secreted via the T3SS1, plays a major role in the T3SS1-dependent cytotoxicity of *V. parahaemolyticus*. However, the mechanism by which VepA is involved in T3SS1-dependent cytotoxicity is unknown. Here, we found that protein transfection of VepA into HeLa cells resulted in cell death, indicating that VepA alone is cytotoxic. The ectopic expression of VepA in yeast *Saccharomyces cerevisiae* interferes with yeast growth, indicating that VepA is also toxic in yeast. A yeast genome-wide screen identified the yeast gene *VMA3* as essential for the growth inhibition of yeast by VepA. Although *VMA3* encodes subunit c of the vacuolar H^+-ATPase (V-ATPase), the toxicity of VepA was independent of the function of V-ATPases. In HeLa cells, knockdown of V-ATPase subunit c decreased VepA-mediated cytotoxicity. We also demonstrated that VepA interacted with V-ATPase subunit c, whereas a carboxyl-terminally truncated mutant of VepA (VepAΔC), which does not show toxicity, did not. During infection, lysosomal contents leaked into the cytosol, revealing that lysosomal membrane permeabilization occurred prior to cell lysis. In a cell-free system, VepA was sufficient to induce the release of cathepsin D from isolated lysosomes. Therefore, our data suggest that the bacterial effector VepA targets subunit c of V-ATPase and induces the rupture of host cell lysosomes and subsequent cell death.

3.1843 Alteration of EGFR Spatiotemporal Dynamics Suppresses Signal Transduction
Turk, H.F., Barhoumi, R. and Chapkin, R.S.
*PloS One, **7(6)**, e39682 (2012)*

The epidermal growth factor receptor (EGFR), which regulates cell growth and survival, is integral to colon tumorigenesis. Lipid rafts play a role in regulating EGFR signaling, and docosahexaenoic acid (DHA) is known to perturb membrane domain organization through changes in lipid rafts. Therefore, we investigated the mechanistic link between EGFR function and DHA. Membrane incorporation of DHA into immortalized colonocytes altered the lateral organization of EGFR. DHA additionally increased EGFR phosphorylation but paradoxically suppressed downstream signaling. Assessment of the EGFR-Ras-ERK1/2 signaling cascade identified Ras GTP binding as the locus of the DHA-induced disruption of signal transduction. DHA also antagonized EGFR signaling capacity by increasing receptor internalization and degradation. DHA suppressed cell proliferation in an EGFR-dependent manner, but cell proliferation could be partially rescued by expression of constitutively active Ras. Feeding chronically-inflamed, carcinogen-injected C57BL/6 mice a fish oil containing diet enriched in DHA recapitulated the effects on the EGFR signaling axis observed in cell culture and additionally suppressed tumor formation. We conclude that DHA-induced alteration in both the lateral and subcellular localization of EGFR culminates in the suppression of EGFR downstream signal transduction, which has implications for the molecular basis of colon cancer prevention by DHA.

3.1844 A Role for Dendritic Translation of CaMKIIα mRNA in Olfactory Plasticity
Neant-Fery, M., Peres, E., Nashrallah, C., Kessner, M., Gribaudo, S., Greer, C., Didier, A., trembleau, A. and Caille, I.
*PloS One, **7(6)**, e40133 (2012)*

Local protein synthesis in dendrites contributes to the synaptic modifications underlying learning and memory. The mRNA encoding the α subunit of the calcium/calmodulin dependent Kinase II (CaMKIIα) is dendritically localized and locally translated. A role for CaMKIIα local translation in hippocampus-dependent memory has been demonstrated in mice with disrupted CaMKIIα dendritic translation, through deletion of CaMKIIα 3′UTR. We studied the dendritic localization and local translation of CaMKIIα in the mouse olfactory bulb (OB), the first relay of the olfactory pathway, which exhibits a high level of plasticity in response to olfactory experience. CaMKIIα is expressed by granule cells (GCs) of the OB. Through *in situ* hybridization and synaptosome preparation, we show that CaMKIIα mRNA is transported in GC dendrites, synaptically localized and might be locally translated at GC synapses. Increases in the synaptic localization of CaMKIIα mRNA and protein in response to brief exposure to new odors demonstrate that they are activity-dependent processes. The activity-induced dendritic transport of CaMKIIα mRNA can be inhibited by an NMDA receptor antagonist and mimicked by an NMDA receptor agonist. Finally, in mice devoid of CaMKIIα 3′UTR, the dendritic localization of CaMKIIα mRNA is disrupted in the OB and olfactory associative learning is severely impaired. Our studies thus reveal a new functional modality for CaMKIIα local translation, as an essential determinant of olfactory plasticity.

3.1845 The Permeability of Reconstituted Nuclear Pores Provides Direct Evidence for the Selective Phase Model

Hülsmann, B.B., Labokha, A.A. and Görlich, D.
*Cell, **150(4)**, 738-751 (2012)*

Nuclear pore complexes (NPCs) maintain a permeability barrier between the nucleus and the cytoplasm through FG-repeat-containing nucleoporins (Nups). We previously proposed a "selective phase model" in which the FG repeats interact with one another to form a sieve-like barrier that can be locally disrupted by the binding of nuclear transport receptors (NTRs), but not by inert macromolecules, allowing selective passage of NTRs and associated cargo. Here, we provide direct evidence for this model in a physiological context. By using NPCs reconstituted from *Xenopus laevis* egg extracts, we show that Nup98 is essential for maintaining the permeability barrier. Specifically, the multivalent cohesion between FG repeats is required, including cohesive FG repeats close to the anchorage point to the NPC scaffold. Our data exclude alternative models that are based solely on an interaction between the FG repeats and NTRs and indicate that the barrier is formed by a sieve-like FG hydrogel.

3.1846 Medulloblastoma Exosome Proteomics Yield Functional Roles for Extracellular Vesicles

Epple, L.M., Griffiths, S.G., Dechkovskaia, A.M., Dusto, N.L., White, J., Ouellette, R.J., Anchordoquy, T.J., Bemis, L.T. and Graner, M.W.
*PloS One, **7(7)**, e42064 (2012)*

Medulloblastomas are the most prevalent malignant pediatric brain tumors. Survival for these patients has remained largely the same for approximately 20 years, and our therapies for these cancers cause significant health, cognitive, behavioral and developmental sequelae for those who survive the tumor and their treatments. We obviously need a better understanding of the biology of these tumors, particularly with regard to their migratory/invasive behaviors, their proliferative propensity, and their abilities to deflect immune responses. Exosomes, virus-sized membrane vesicles released extracellularly from cells after formation in, and transit thru, the endosomal pathway, may play roles in medulloblastoma pathogenesis but are as yet unstudied in this disease. Here we characterized exosomes from a medulloblastoma cell line with biochemical and proteomic analyses, and included characterization of patient serum exosomes. Further scrutiny of the proteomic data suggested functional properties of the exosomes that are relevant to medulloblastoma tumor biology, including their roles as proliferation stimulants, their activities as attractants for tumor cell migration, and their immune modulatory impacts on lymphocytes. Aspects of this held true for exosomes from other medulloblastoma cell lines as well. Additionally, pathway analyses suggested a possible role for the transcription factor hepatocyte nuclear factor 4 alpha (HNF4A); however, inhibition of the protein's activity actually increased D283MED cell proliferation/clonogenecity, suggesting that HNF4A may act as a tumor suppressor in this cell line. Our work demonstrates that relevant functional properties of exosomes may be derived from appropriate proteomic analyses, which translate into mechanisms of tumor pathophysiology harbored in these extracellular vesicles.

3.1847 Association between Tetrodotoxin Resistant Channels and Lipid Rafts Regulates Sensory Neuron Excitability

Pristera, A., Baker, M.D. and Okuse, K.
*PloS One, **7(8)**, e40079 (2012)*

Voltage-gated sodium channels (VGSCs) play a key role in the initiation and propagation of action potentials in neurons. $Na_V1.8$ is a tetrodotoxin (TTX) resistant VGSC expressed in nociceptors, peripheral small-diameter neurons able to detect noxious stimuli. $Na_V1.8$ underlies the vast majority of sodium currents during action potentials. Many studies have highlighted a key role for $Na_V1.8$ in inflammatory and chronic pain models. Lipid rafts are microdomains of the plasma membrane highly enriched in cholesterol and sphingolipids. Lipid rafts tune the spatial and temporal organisation of proteins and lipids on the plasma membrane. They are thought to act as platforms on the membrane where proteins and lipids can be trafficked, compartmentalised and functionally clustered. In the present study we investigated $Na_V1.8$ sub-cellular localisation and explored the idea that it is associated with lipid rafts in nociceptors. We found that $Na_V1.8$ is distributed in clusters along the axons of DRG neurons *in vitro* and *ex vivo*. We also demonstrated, by biochemical and imaging studies, that $Na_V1.8$ is associated with lipid rafts along the sciatic nerve *ex vivo* and in DRG neurons *in vitro*. Moreover, treatments with methyl-β-cyclodextrin (MβCD) and 7-ketocholesterol (7KC) led to the dissociation between rafts and $Na_V1.8$. By calcium imaging we demonstrated that the lack of association between rafts and $Na_V1.8$ correlated with impaired neuronal excitability, highlighted by a reduction in the number of neurons able to conduct mechanically-

and chemically-evoked depolarisations. These findings reveal the sub-cellular localisation of $Na_V1.8$ in nociceptors and highlight the importance of the association between $Na_V1.8$ and lipid rafts in the control of nociceptor excitability.

3.1848 Respiratory Syncytial Virus Assembles into Structured Filamentous Virion Particles Independently of Host Cytoskeleton and Related Proteins
Shaikh, F.Y., Utley, T.J., Craven, R.E., Rogers, M.C., Lapierre, L.A., Goldenring, J.R. and Crowe Jr. J.E.
*PloS One, **7(7)**, e40826 (2012)*

Respiratory syncytial virus (RSV) is a single-stranded RNA virus that assembles into viral filaments at the cell surface. Virus assembly often depends on the ability of a virus to use host proteins to accomplish viral tasks. Since the fusion protein cytoplasmic tail (FCT) is critical for viral filamentous assembly, we hypothesized that host proteins important for viral assembly may be recruited by the FCT. Using a yeast two-hybrid screen, we found that filamin A interacted with FCT, and mammalian cell experiments showed it localized to viral filaments but did not affect viral replication. Furthermore, we found that a number of actin-associated proteins also were excluded from viral filaments. Actin or tubulin cytoskeletal rearrangement was not necessary for F trafficking to the cell surface or for viral assembly into filaments, but was necessary for optimal viral replication and may be important for anchoring viral filaments. These findings suggest that RSV assembly into filaments occurs independently of actin polymerization and that viral proteins are the principal drivers for the mechanical tasks involved with formation of complex, structured RSV filaments at the host cell plasma membrane.

3.1849 Characterization of the Early Steps of Human Parvovirus B19 Infection
Quattrocchi, S., Ruprecht, N., Bönsch, C., Bieli, S., Zürcher, C., Boller, K., Kempf, C. and Ros, C.
*J. Virol., **86(17)**, 9274-9284 (2012)*

The early steps of human parvovirus B19 (B19V) infection were investigated in UT7/Epo cells. B19V and its receptor globoside (Gb4Cer) associate with lipid rafts, predominantly of the noncaveolar type. Pharmacological disruption of the lipid rafts inhibited infection when the drug was added prior to virus attachment but not after virus uptake. B19V is internalized by clathrin-dependent endocytosis and spreads rapidly throughout the endocytic pathway, reaching the lysosomal compartment within minutes, where a substantial proportion is degraded. B19V did not permeabilize the endocytic vesicles, indicating a mechanism of endosomal escape without apparent membrane damage. Bafilomycin A_1 (BafA1) and NH_4Cl, which raise endosomal pH, blocked the infection by preventing endosomal escape, resulting in a massive accumulation of capsids in the lysosomes. In contrast, in the presence of chloroquine (CQ), the transfer of incoming viruses from late endosomes to lysosomes was prevented; the viral DNA was not degraded; and the infection was boosted. In contrast to the findings for untreated or BafA1-treated cells, the viral DNA was progressively associated with the nucleus in CQ-treated cells, reaching a plateau by 3 h postinternalization, a time coinciding with the initiation of viral transcription. At this time, more than half of the total intracellular viral DNA was associated with the nucleus; however, the capsids remained extranuclear. Our studies provide the first insight into the early steps of B19V infection and reveal mechanisms involved in virus uptake, endocytic trafficking, and nuclear penetration.

3.1850 ADP ribosylation adapts an ER chaperone response to short-term fluctuations in unfolded protein load
Chambers, J.E., Petrova, K., Tomba, G., Vendruscolo, M. and Ron, D.
*J. Cell Biol., **198(3)**, 371-385 (2012)*

Gene expression programs that regulate the abundance of the chaperone BiP adapt the endoplasmic reticulum (ER) to unfolded protein load. However, such programs are slow compared with physiological fluctuations in secreted protein synthesis. While searching for mechanisms that fill this temporal gap in coping with ER stress, we found elevated levels of adenosine diphosphate (ADP)–ribosylated BiP in the inactive pancreas of fasted mice and a rapid decline in this modification in the active fed state. ADP ribosylation mapped to Arg470 and Arg492 in the substrate-binding domain of hamster BiP. Mutations that mimic the negative charge of ADP-ribose destabilized substrate binding and interfered with interdomain allosteric coupling, marking ADP ribosylation as a rapid posttranslational mechanism for reversible inactivation of BiP. A kinetic model showed that buffering fluctuations in unfolded protein load with a recruitable pool of inactive chaperone is an efficient strategy to minimize both aggregation and costly degradation of unfolded proteins.

3.1851 Calpains mediate epithelial-cell death during mammary gland involution: mitochondria and lysosomal destabilization

Arnandis, T., Ferrer-Vicens, I., Garcia-Trevijano, E.R., Miralles, V.J., Garcia, C., Torres, L., Vina, J.R. and Zaragoza, R.

Cell Death & Differentiation, ***19(9)***, *1536-1548 (2012)*

Our aim was to elucidate the physiological role of calpains (CAPN) in mammary gland involution. Both CAPN-1 and -2 were induced after weaning and its activity increased in isolated mitochondria and lysosomes. CAPN activation within the mitochondria could trigger the release of cytochrome *c* and other pro-apoptotic factors, whereas in lysosomes it might be essential for tissue remodeling by releasing cathepsins into the cytosol. Immunohistochemical analysis localized CAPNs mainly at the luminal side of alveoli. During weaning, CAPNs translocate to the lysosomes processing membrane proteins. To identify these substrates, lysosomal fractions were treated with recombinant CAPN and cleaved products were identified by 2D-DIGE. The subunit b_2 of the v-type H^+ ATPase is proteolyzed and so is the lysosomal-associated membrane protein 2a (LAMP2a). Both proteins are also cleaved *in vivo*. Furthermore, LAMP2a cleavage was confirmed *in vitro* by addition of CAPNs to isolated lysosomes and several CAPN inhibitors prevented it. Finally, *in vivo* inhibition of CAPN1 in 72-h-weaned mice decreased LAMP2a cleavage. Indeed, calpeptin-treated mice showed a substantial delay in tissue remodeling and involution of the mammary gland. These results suggest that CAPNs are responsible for mitochondrial and lysosomal membrane permeabilization, supporting the idea that lysosomal-mediated cell death is a new hallmark of mammary gland involution.

3.1852 Caveolae and propofol effects on airway smooth muscle

Grim, K.J., Abcejo, A.J., Barnes, A., Sathish, V., Smelter, D.F., Ford, G.C., Thompson, M.A., prakash, Y.S. and Pabelick, C.M.

Br. J. Anaesth., ***109(3)***, *444-453 (2012)*

Background The i.v. anaesthetic propofol produces bronchodilatation. Airway relaxation involves reduced intracellular Ca^{2+} ($[Ca^{2+}]_i$) in airway smooth muscle (ASM) and lipid rafts (caveolae), and constitutional caveolin proteins regulate $[Ca^{2+}]_i$. We postulated that propofol-induced bronchodilatation involves caveolar disruption.
Methods Caveolar fractions of human ASM cells were tested for propofol content. $[Ca^{2+}]_i$ responses of ASM cells loaded with fura-2 were performed in the presence of 10 μM histamine with and without clinically relevant concentrations of propofol (10 and 30 μM and intralipid control). Effects on sarcoplasmic reticulum (SR) Ca^{2+} release were evaluated in zero extracellular Ca^{2+} using the blockers Xestospongin C and ryanodine. Store-operated Ca^{2+} entry (SOCE) after SR depletion was evaluated using established techniques. The role of caveolin-1 in the effect of propofol was tested using small interference RNA (siRNA) suppression. Changes in intracellular signalling cascades relevant to $[Ca^{2+}]_i$ and force regulation were also evaluated.
Results Propofol was present in ASM caveolar fractions in substantial concentrations. Exposure to 10 or 30 μM propofol form decreased $[Ca^{2+}]_i$ peak (but not plateau) responses to histamine by ~40%, an effect persistent in zero extracellular Ca^{2+}. Propofol effects were absent in caveolin-1 siRNA-transfected cells. Inhibition of ryanodine receptors prevented propofol effects on $[Ca^{2+}]_i$, while propofol blunted $[Ca^{2+}]_i$ responses to caffeine. Propofol reduced SOCE, an effect also prevented by caveolin-1 siRNA. Propofol effects were associated with decreased caveolin-1 expression and extracellular signal-regulated kinase phosphorylation.
Conclusions These novel data suggest a role for caveolae (specifically caveolin-1) in propofol-induced bronchodilatation. Due to its lipid nature, propofol may transiently disrupt caveolar regulation, thus altering ASM $[Ca^{2+}]_i$.

3.1853 T-Cadherin Is an Auxiliary Negative Regulator of EGFR Pathway Activity in Cutaneous Squamous Cell Carcinoma: Impact on Cell Motility

Kyriakakis, E., Maslova, K., Philippova, M., Pfaff, D., Joshi, M.B., Buechner, S.A., Erne, P. and resink, T.J.

J. Invest. Dermatol., ***132(9)***, *2275-2285 (2012)*

Genetic and epigenetic studies in different cancers, including cutaneous carcinomas, have implicated T-cadherin (T-cad) as a tumor suppressor. Immunohistochemical and *in vitro* studies have suggested that T-cad loss promotes incipient invasiveness in cutaneous squamous cell carcinoma (SCC). Molecular

mechanisms are unknown. This study found that the main consequence of T-cad silencing in SCC is facilitation of ligand-dependent EGFR activation, whereas T-cad overexpression impedes EGFR activation. Gain- and loss-of-function studies in A431 SCC cells demonstrate T-cad-controlled responsiveness to EGF with respect to pharmacological inhibition of EGFR and to diverse signaling and functional events of the EGFR activation cascade (EGFR phosphorylation, internalization, nuclear translocation, cell retraction/de-adhesion, motility, invasion, integrin β1, and Rho small GTPases such as RhoA, Rac1, and Cdc42 activation). Further, T-cad modulates the EGFR pathway activity by influencing membrane compartmentalization of EGFR; T-cad upregulation promotes retention of EGFR in lipid rafts, whereas T-cad silencing releases EGFR from this compartment, rendering EGFR more accessible to ligand stimulation. This study reveals a mechanism for fine-tuning of EGFR activity in SCC, whereby T-cad represents an auxiliary "negative" regulator of the EGFR pathway, which impacts invasion-associated behavioral responses of SCC to EGF. This action of T-cad in SCC may serve as a paradigm explaining other malignancies displaying concomitant T-cad loss and enhanced EGFR activity.

3.1854 Evidence for a dual function of EphB4 as tumor promoter and suppressor regulated by the absence or presence of the ephrin-B2 ligand
Rutkowski, R., Mertens-Walker, I., Lisle, J.E., herington, A.C. and Stephenson, S-A.
Int. J. Cancer, ***131(5)****, E614-E624 (2012)*

Overexpression of the receptor tyrosine kinase EphB4 is common in epithelial cancers and linked to tumor progression by promoting angiogenesis, increasing survival and facilitating invasion and migration. However, other studies have reported loss of EphB4 suggesting a tumor suppressor function in some cancers. These opposing roles may be regulated by (*i*) the presence of the primary ligand ephrin-B2 that regulates pathways involved in tumor suppression or (*ii*) the absence of ephrin-B2 that allows EphB4 signaling *via* ligand-independent pathways that contribute to tumor promotion. To explore this theory, EphB4 was overexpressed in the prostate cancer cell line 22Rv1 and the mammary epithelial cell line MCF-10A. Overexpressed EphB4 localized to lipid-rich regions of the plasma membrane and confirmed to be ligand-responsive as demonstrated by increased phosphorylation of ERK1/2 and internalization. EphB4 overexpressing cells demonstrated enhanced anchorage-independent growth, migration and invasion, all characteristics associated with an aggressive phenotype, and therefore supporting the hypothesis that overexpressed EphB4 facilitates tumor promotion. Importantly, these effects were reversed in the presence of ephrin-B2 which led to a reduction in EphB4 protein levels, demonstrating that ligand-dependent signaling is tumor suppressive. Furthermore, extended ligand stimulation caused a significant decrease in proliferation that correlated with a rise in caspase-3/7 and -8 activities. Together, these results demonstrate that overexpression of EphB4 confers a transformed phenotype in the case of MCF-10A cells and an increased metastatic phenotype in the case of 22Rv1 cancer cells and that both phenotypes can be restrained by stimulation with ephrin-B2, in part by reducing EphB4 levels.

3.1855 Syndecan–syntenin–ALIX regulates the biogenesis of exosomes
Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortyere, F., Coomans, C., Vermeiren, E., Zimmermann, P. and David, G.
Nature Cell Biol., ***14(7)****, 677-685 (2012)*

The biogenesis of exosomes, small secreted vesicles involved in signalling processes, remains incompletely understood. Here, we report evidence that the syndecan heparan sulphate proteoglycans and their cytoplasmic adaptor syntenin control the formation of exosomes. Syntenin interacts directly with ALIX through LYPX(n)L motifs, similarly to retroviral proteins, and supports the intraluminal budding of endosomal membranes. Syntenin exosomes depend on the availability of heparan sulphate, syndecans, ALIX and ESCRTs, and impact on the trafficking and confinement of FGF signals. This study identifies a key role for syndecan–syntenin–ALIX in membrane transport and signalling processes.

3.1856 Regulation of Cell Migration by Sphingomyelin Synthases: Sphingomyelin in Lipid Rafts Decreases Responsiveness to Signaling by the CXCL12/CXCR4 Pathway
Asano, S., Kitani, K., Taniguchi, M., Hashimoto, M., Zama, K., Mitsutake, S., Igarashi, Y., Takeya, H., Kigawa, J., Hayashi, A., Umehara, H. and Okazaki, T.
Mol. Cell. Biol., ***32(16)****, 3242-3252 (2012)*

Sphingomyelin synthase (SMS) catalyzes the formation of sphingomyelin, a major component of the plasma membrane and lipid rafts. To investigate the role of SMS in cell signaling and migration induced by binding of the chemokine CXCL12 to CXCR4, we used mouse embryonic fibroblasts deficient in *SMS1*

and/or *SMS2* and examined the effects of SMS deficiency on cell migration. SMS deficiency promoted cell migration through a CXCL12/CXCR4-dependent signaling pathway involving extracellular signal-regulated kinase (ERK) activation. In addition, *SMS1*/*SMS2* double-knockout cells had heightened sensitivity to CXCL12, which was significantly suppressed upon transfection with the *SMS1* or *SMS2* gene or when they were treated with exogenous sphingomyelin but not when they were treated with the SMS substrate ceramide. Notably, SMS deficiency facilitated relocalization of CXCR4 to lipid rafts, which form platforms for the regulation and transduction of receptor-mediated signaling. Furthermore, we found that SMS deficiency potentiated CXCR4 dimerization, which is required for signal transduction. This dimerization was significantly repressed by sphingomyelin treatment. Collectively, our data indicate that SMS-derived sphingomyelin lowers responsiveness to CXCL12, thereby reducing migration induced by this chemokine. Our findings provide the first direct evidence for an involvement of SMS-generated sphingomyelin in the regulation of cell migration.

3.1857 R-SNARE ykt6 resides in membrane-associated protease-resistant protein particles and modulates cell cycle progression when over-expressed

Thayanidhi, N., Liang, Y., Hasegawa, H., Nycz, D.C., oorschot, V., Klumperman, J. and Hay, J.C.
Biol. Cell, ***104(7)***, *397-417 (2012)*

Background information
The arginine-type soluble N-ethylmaleimide-sensitive factor attachment protein receptor (R-SNARE) ykt6 possesses several atypical properties including selective high expression in neurons, a lipidated C-terminus, localization to punctae that do not correspond with known endomembrane markers, a potent ability to protect the secretory pathway from alpha-synuclein over-expression and specific up-regulation in tumors. We have followed up on several of these features that together suggest nontraditional SNARE structures and functions.
Results
A significant portion of ykt6 in PC12 cells was found in a protease-resistant state suggestive of a large complex or aggregate. Other endoplasmic reticulum/Golgi SNAREs were not protease resistant, demonstrating that SNARE complexes *per se* did not cause protease resistance. Mutagenesis indicated that lipidation of the ykt6 C-terminus was also not involved, implicating its longin domain in particle formation. Immunogold electron microscopy revealed ykt6 labeling of ~100 nm electron densities associated with diverse membranes. Density gradient analysis of the protease-resistant structures confirmed their tight association with membranes. Since excess ykt6 has been correlated with tumorigenesis, we tested whether ykt6 over-expression in normal rat kidney cells that normally express little ykt6 affected the cell cycle. Ykt6 over-expression was found to result in altered cell division cycles as evidenced by significantly smaller cells, a higher mitotic index and increased DNA synthesis. Mutagenesis studies dis-correlated SNARE function with the cell cycle effects; instead, the cell cycle effects correlated better with ykt6 properties related to the longin domain or particle formation.
Conclusions
The ykt6 particles/aggregates may represent ykt6 engaged in a non-SNARE function(s) or else nonfunctional, stored and/or excess ykt6. Whether the particulate ykt6 structures represent a means of buffering the apparent proliferative activity or are in fact mechanistically related to this activity will be of future interest in neuroscience and cancer biology.

3.1858 Identification of Autophagosome-associated Proteins and Regulators by Quantitative Proteomic Analysis and Genetic Screens

Dengjel, J., Høyer-hansden, M., Nielsen, M.O., Eisenberg, T., Harder, L.M., Schandorff, S., Farkas, T., Kirkegaard, T., Becker, A.C., Schroeder, S., Vanswelow, K., Lundberg, E., Nielsen, M.M., kristensen, A.R., Akimov, V., Bunkenborg, J., Madeo, F., Jäättelä, M. and Andersen, J.S.
Mol. Cell. Proteomics, ***11***, *1-17 (2012)*

Autophagy is one of the major intracellular catabolic pathways, but little is known about the composition of autophagosomes. To study the associated proteins, we isolated autophagosomes from human breast cancer cells using two different biochemical methods and three stimulus types: amino acid deprivation or rapamycin or concanamycin A treatment. The autophagosome-associated proteins were dependent on stimulus, but a core set of proteins was stimulus-independent. Remarkably, proteasomal proteins were abundant among the stimulus-independent common autophagosome-associated proteins, and the activation of autophagy significantly decreased the cellular proteasome level and activity supporting interplay between the two degradation pathways. A screen of yeast strains defective in the orthologs of the human genes encoding for a common set of autophagosome-associated proteins revealed several regulators of

autophagy, including subunits of the retromer complex. The combined spatiotemporal proteomic and genetic data sets presented here provide a basis for further characterization of autophagosome biogenesis and cargo selection.

3.1859 A Mechanism of Intracellular P2X Receptor Activation
Sivaramakrishnan, V. and Fountain, S.J.
J. Biol. Chem., ***287(34)***, *28315-28326 (2012)*

P2X receptors (P2XRs) are ATP-activated calcium-permeable ligand-gated ion channels traditionally viewed as sensors of extracellular ATP during diverse physiological processes including pain, inflammation, and taste. However, in addition to a cell surface residency P2XRs also populate the membranes of intracellular compartments, including mammalian lysosomes, phagosomes, and the contractile vacuole (CV) of the amoeba *Dictyostelium*. The function of intracellular P2XRs is unclear and represents a major gap in our understanding of ATP signaling. Here, we exploit the genetic versatility of *Dictyostelium* to investigate the effects of physiological concentrations of ATP on calcium signaling in isolated CVs. Within the CV, an acidic calcium store, P2XRs are orientated to sense luminal ATP. Application of ATP to isolated vacuoles leads to luminal translocation of ATP and release of calcium. Mechanisms of luminal ATP translocation and ATP-evoked calcium release share common pharmacology, suggesting that they are linked processes. The ability of ATP to mobilize stored calcium is reduced in vacuoles isolated from $P2X_AR$ knock-out amoeba and ablated in cells devoid of P2XRs. Pharmacological inhibition of luminal ATP translocation or depletion of CV calcium attenuates CV function *in vivo*, manifesting as a loss of regulatory cell volume decrease following osmotic swelling. We propose that intracellular P2XRs regulate vacuole activity by acting as calcium release channels, activated by translocation of ATP into the vacuole lumen.

3.1860 High insulin levels are required for FAT/CD36 plasma membrane translocation and enhanced fatty acid uptake in obese Zucker rat hepatocytes
Buque, X., Cano, A., Miquilena-Colina, M.E., Garcia-Monzon, C., Ochoa, B. and Aspichueta, P.
Am. J. Physiol. Endocrinol. Metab., 303, E504-E514 (2012)

In myocytes and adipocytes, insulin increases fatty acid translocase (FAT)/CD36 translocation to theplasma membrane (PM), enhancing fatty acid (FA) uptake. Evidence links increased hepatic FAT/CD36 protein amount and gene expression with hyperinsulinemia in animal models and patients with fatty liver, but whether insulin regulates FAT/CD36 expression, amount, distribution, and function in hepatocytes is currently unknown. To investigate this, FAT/CD36 protein content in isolated hepatocytes, subfractions of organelles, and density-gradient isolated membrane subfractions was analyzed in obese and lean Zucker rats by Western blotting in liver sections by immunohistochemistry and in hepatocytes by immunocytochemistry. The uptake of oleate and oleate incorporation into lipids were assessed in hepatocytes at short time points (30–600 s). We found that FAT/CD36 protein amount at the PM was higher in hepatocytes from obese rats than from lean controls. In obese rat hepatocytes, decreased cytoplasmatic content of FAT/CD36 and redistribution from low- to middle- to middle- to high-density subfractions of microsomes were found. Hallmarks of obese Zucker rat hepatocytes were increased amount of FAT/CD36 protein at the PM and enhanced FA uptake and incorporation into triglycerides, which were maintained only when exposed to hyperinsulinemic conditions (80 mU/l). In conclusion, high insulin levels are required for FAT/CD36 translocation to the PM in obese rat hepatocytes to enhance FA uptake and triglyceride synthesis. These results suggest that the hyperinsulinemia found in animal models and patients with insulin resistance and fatty liver might contribute to liver fat accumulation by inducing FAT/CD36 functional presence at the PM of hepatocytes.

3.1861 GTP-Binding-Defective ARL4D Alters Mitochondrial Morphology and Membrane Potential
Li, C-C., Wu, T-S., Huang, C-F., Jang, L-T., Liu, Y-T., You, S-T., Liou, G-G. and Lee, F-J.S.
PloS One, ***7(8)****, e43552 (2012)*

ARL4D, ARL4A, and ARL4C are closely related members of the ADP-ribosylation factor/ARF-like protein (ARF/ARL) family of GTPases. All three ARL4 proteins contain nuclear localization signals (NLSs) at their C-termini and are primarily found at the plasma membrane, but they are also present in the nucleus and cytoplasm. ARF function and localization depends on their controlled binding and hydrolysis of GTP. Here we show that GTP-binding-defective ARL4D is targeted to the mitochondria, where it affects mitochondrial morphology and function. We found that a portion of endogenous ARL4D and the GTP-binding-defective ARL4D mutant ARL4D(T35N) reside in the mitochondria. The N-terminal

myristoylation of ARL4D(T35N) was required for its localization to mitochondria. The localization of ARL4D(T35N) to the mitochondria reduced the mitochondrial membrane potential ($\Delta\Psi m$) and caused mitochondrial fragmentation. Furthermore, the C-terminal NLS region of ARL4D(T35N) was required for its effect on the mitochondria. This study is the first to demonstrate that the dysfunctional GTP-binding-defective ARL4D is targeted to mitochondria, where it subsequently alters mitochondrial morphology and membrane potential.

3.1862 N-linked glycosylation of dimeric acetylcholinesterase in erythrocytes is essential for enzyme maturation and membrane targeting
Luk, W.K.W., Chen, V.P., Choi, R.C. and Tsim, K.W.K.
FEBS J., ***279(12)***, *3229-3239 (2012)*

Acetylcholinesterase (AChE) is well-known for its cholinergic functions in the nervous system; however, this enzyme is also found in other tissues where its function is still not understood. AChE is synthesized through alternative splicing as splicing variants, with isoforms including read-through ($AChE_R$), tailed ($AChE_T$) and hydrophobic ($AChE_H$). In human erythrocytes, $AChE_H$ is a glycophosphatidylinositol-linked dimer on the plasma membrane. Three N-linked glycosylation sites have been identified in the catalytic domain of human AChE. Here, we investigate the roles of glycosylation in assembly and trafficking of human $AChE_H$. In transfected fibroblasts, expression of $AChE_H$ was able to mimic the function of the dimeric form of AChE on the erythrocyte membrane. A glycan-depleted form was constructed by site-directed mutagenesis. By comparison with the wild-type $AChE_H$, the mutant had a much lower enzymatic activity and a much higher K_m value. In addition, the mutant was dimerized in the endoplasmic reticulum, but was not trafficked to the Golgi apparatus. The results suggest that the glycosylation may affect $AChE_H$ enzymatic activity and trafficking, but not dimer formation. The present findings indicate the significance of N-glycosylation in controlling the biosynthesis of the $AChE_H$ dimer form.

3.1863 NEU4L sialidase overexpression promotes β-catenin signaling in neuroblastoma cells, enhancing stem-like malignant cell growth
Tringali, C., Cirillo, F., Lamorte, G., Papini, N., Anastasia, L., Lupo, B., Silvestri, I., Tettaamanti, G. and Venerando, B.
Int. J. Cancer, ***131(8)***, *1768-1778 (2012)*

Neuroblastoma (NB) is a frequently lethal tumor that occurs in childhood and originates from embryonic neural crest cells. The malignant and aggressive phenotype of NB is strictly related to the deregulation of pivotal pathways governing the proliferation/differentiation status of neural crest precursor cells, such as *MYCN*, Delta/Notch and Wnt/β-catenin (*CTNNB1*) signaling. In this article, we demonstrate that sialidase NEU4 long (NEU4L) influences the differentiation/proliferation behavior of NB SK-N-BE cells by determining hyperactivation of the Wnt/β-catenin signaling pathway. *NEU4L* overexpression in SK-N-BE cells induced significant increases in active, nonphosphorylated β-catenin content, β-catenin/TCF transcriptional activity and β-catenin gene target expression including *MYCN*, *MYC*, *CCND2* (cyclin D2) and *CDC25A*. In turn, these molecular features strongly modified the behavior of *NEU4L* SK-N-BE overexpressing cells, promoting the following: (1) an enhanced proliferation rate, mainly due to a faster transition from G1 to S phase in the cell cycle; (2) a more undifferentiated cell phenotype, which was similar to stem-like NB cells and possibly mediated by an increase of the expression of the pluripotency genes, *MYC*, *NANOG*, *OCT-4*, *CD133* and *NES* (nestin); (3) the failure of NB cell differentiation after serum withdrawal. The molecular link between NEU4L and Wnt/β-catenin signaling appeared to rely most likely on the capability of the enzyme to modify the sialylation level of cell glycoproteins. These findings could provide a new candidate for therapeutic treatment.

3.1864 The Role of Cholesterol in UV Light B-induced Apoptosis
George, K.S., Elyassaki, W., Wu, Q. and Wu, S.
Photchem. Photobiol., ***88(5)***, *1191-1197 (2012)*

Modification of major lipid raft components, such as cholesterol and ceramide, plays a role in regulation of programmed cell death under various stimuli. However, the relationship between cholesterol level modification and the activation of apoptotic signaling cascades upon UVB light has not been established. In this report, we demonstrate that upon UVB irradiation cholesterol levels in membrane rafts of skin cells increase, which leads to Fas-receptor (Fas) aggregation in the rafts. Utilizing a continuous velocity floatation technique, we show that Fas accumulated in the lipid rafts of human melanoma M624 cells after UVB irradiation. The subsequent events of death-inducing signaling complex formation were also detected

in the lipid raft fractions. Depletion of cholesterol by methyl-β-cyclodextrin reduces Fas aggregation, while overloading increases. Disruption of lipid rafts also prevents Fas death domain-associated protein (Daxx) from dissociating from Fas in the lipid rafts, which is accompanied with a reduced apoptotic, but increased nonapoptotic death of UVB-irradiated human keratinocytes, HaCaT cells. Results indicate that cholesterol located in the plasma membrane of skin cells is required for lipid raft domain formation and activation of UVB-induced apoptosis.

3.1865 P-glycoprotein trafficking at the blood–brain barrier altered by peripheral inflammatory hyperalgesia

McCaffrey, G., Staatz, W.D., Sanchez-Covarrubias, L., Finch, J., DeMarco, K., Laracuente, M-L., Ronaldson, P.T. and Davis, T.P.
J. Neurochem., ***122(5)***, *962-975 (2012)*

P-glycoprotein (ABCB1/MDR1, EC 3.6.3.44), the major efflux transporter at the blood–brain barrier (BBB), is a formidable obstacle to CNS pharmacotherapy. Understanding the mechanism(s) for increased P-glycoprotein activity at the BBB during peripheral inflammatory pain is critical in the development of novel strategies to overcome the significant decreases in CNS analgesic drug delivery. In this study, we employed the λ-carrageenan pain model (using female Sprague–Dawley rats), combined with confocal microscopy and subcellular fractionation of cerebral microvessels, to determine if increased P-glycoprotein function, following the onset of peripheral inflammatory pain, is associated with a change in P-glycoprotein trafficking which leads to pain-induced effects on analgesic drug delivery. Injection of λ-carrageenan into the rat hind paw induced a localized, inflammatory pain (hyperalgesia) and simultaneously, at the BBB, a rapid change in colocalization of P-glycoprotein with caveolin-1, a key scaffolding/trafficking protein. Subcellular fractionation of isolated cerebral microvessels revealed that the bulk of P-glycoprotein constitutively traffics to membrane domains containing high molecular weight, disulfide-bonded P-glycoprotein-containing structures that cofractionate with membrane domains enriched with monomeric and high molecular weight, disulfide-bonded, caveolin-1-containing structures. Peripheral inflammatory pain promoted a dynamic redistribution between membrane domains of P-glycoprotein and caveolin-1. Disassembly of high molecular weight P-glycoprotein-containing structures within microvascular endothelial luminal membrane domains was accompanied by an increase in ATPase activity, suggesting a potential for functionally active P-glycoprotein. These results are the first observation that peripheral inflammatory pain leads to specific structural changes in P-glycoprotein responsible for controlling analgesic drug delivery to the CNS.

3.1866 Vaccinia Virus Virion Membrane Biogenesis Protein A11 Associates with Viral Membranes in a Manner That Requires the Expression of Another Membrane Biogenesis Protein, A6

Wu, X., Meng, X., Yan, B., Rose, L., Deng, J. and Xiang, Y.
J. Virol., ***86(20)***, *11276-11286 (2012)*

A group of vaccinia virus (VACV) proteins, including A11, L2, and A6, are required for biogenesis of the primary envelope of VACV, specifically, for the acquisition of viral membrane precursors. However, the interconnection among these proteins is unknown and, with the exception of L2, the connection of these proteins with membranes is also unknown. In this study, prompted by the findings that A6 coprecipitated A11 and that the cellular distribution of A11 was dramatically altered by repression of A6 expression, we studied the localization of A11 in cells by using immunofluorescence and cell fractionation analysis. A11 was found to associate with membranes and colocalize with virion membrane proteins in viral replication factories during normal VACV replication. A11 partitioned almost equally between the detergent and aqueous phases upon Triton X-114 phase separation, demonstrating an intrinsic affinity with lipids. However, in the absence of infection or VACV late protein synthesis, A11 did not associate with cellular membranes. Furthermore, when A6 expression was repressed, A11 did not colocalize with any viral membrane proteins or associate with membranes. In contrast, when virion envelope formation was blocked at a later step by repression of A14 expression or by rifampin treatment, A11 colocalized with virion membrane proteins in the factories. Altogether, our data showed that A11 associates with viral membranes during VACV replication, and this association requires A6 expression. This study provides a physical connection between A11 and viral membranes and suggests that A6 regulates A11 membrane association.

3.1867 Identification of Transmembrane Protein 134 as a Novel LMP1-Binding Protein by Using Bimolecular Fluorescence Complementation and an Enhanced Retroviral Mutagen

Talaty, P., Emery, A., Holthusen, K. and Everly Jr., D.N.
J. Virol., ***86(20)***, *11345-11355 (2012)*

Latent membrane protein 1 (LMP1) of Epstein-Barr virus induces constitutive signaling in infected cells. LMP1 signaling requires oligomerization of LMP1 via its transmembrane domain, localization to lipid rafts in the membrane, and association of the LMP1 cytoplasmic domain to adaptor proteins, such as the tumor necrosis factor receptor-associated factors (TRAFs). Protein complementation is a novel technique to examine protein-protein interaction through the assembly of functional fluorescent proteins or enzymes from inactive fragments. A previous study in our lab demonstrated the use of bimolecular fluorescence complementation (BiFC) to study the assembly of the LMP1 signaling complexes within the plasma membrane of mammalian cells. In the present study, LMP1 was used as bait in a genome-wide BiFC screen with an enhanced retroviral mutagen to identify new LMP1-binding proteins. Our screen identified a novel LMP1-binding protein, transmembrane protein 134 (Tmem134). Tmem134 is a candidate oncogene that is amplified in breast cancer cell lines. Binding, colocalization, and cofractionation between LMP1 and Tmem134 were confirmed. Finally, Tmem134 affected LMP1-induced NF-κB induction. Together, these data suggest that BiFC is a unique and novel platform to identify proteins recruited to the LMP1-signaling complex.

3.1868 Loss of Retinoschisin (RS1) Cell Surface Protein in Maturing Mouse Rod Photoreceptors Elevates the Luminance Threshold for Light-Driven Translocation of Transducin But Not Arrestin
Ziccardi, L., Vijayasarathy, C., Bush, R.A. and Sieving, P.A.
*J. Neurosci., **32(38)**, 13010-13021 (2012)*

Loss of retinoschisin (RS1) in *Rs1* knock-out (*Rs1*–KO) retina produces a post-photoreceptor phenotype similar to X-linked retinoschisis in young males. However, *Rs1* is expressed strongly in photoreceptors, and *Rs1*–KO mice have early reduction in the electroretinogram a-wave. We examined light-activated transducin and arrestin translocation in young *Rs1*–KO mice as a marker for functional abnormalities in maturing rod photoreceptors. We found a progressive reduction in luminance threshold for transducin translocation in wild-type (WT) retinas between postnatal days P18 and P60. At P21, the threshold in *Rs1*–KO retinas was 10-fold higher than WT, but it decreased to <2.5-fold higher by P60. Light-activated arrestin translocation and re-translocation of transducin in the dark were not affected. *Rs1*–KO rod outer segment (ROS) length was significantly shorter than WT at P21 but was comparable with WT at P60. These findings suggested a delay in the structural and functional maturation of *Rs1*–KO ROS. Consistent with this, transcription factors CRX and NRL, which are fundamental to maturation of rod protein expression, were reduced in ROS of *Rs1*–KO mice at P21 but not at P60. Expression of transducin was 15–30% lower in P21 *Rs1*–KO ROS and transducin GTPase hydrolysis was nearly twofold faster, reflecting a 1.7- to 2.5-fold increase in RGS9 (regulator of G-protein signaling) level. Transduction protein expression and activity levels were similar to WT at P60. Transducin translocation threshold elevation indicates photoreceptor functional abnormalities in young *Rs1*–KO mice. Rapid reduction in threshold coupled with age-related changes in transduction protein levels and transcription factor expression are consistent with delayed maturation of *Rs1*–KO photoreceptors.

3.1869 Assessing Heterogeneity of Peroxisomes: Isolation of Two Subpopulations from Rat Liver
Islinger, M., Abdolzade-Bavil, A., Liebler, S., Weber, G. and Völkl, A.
*Methods in Mol. Biol., **909**, 83-96 (2012)*

Peroxisomes exhibit a heterogeneous morphological appearance in rat liver tissue. In this respect, the isolation and subsequent biochemical characterization of peroxisome species from different subcellular prefractions should help to solve the question of whether peroxisomes indeed diverge into functionally specialized subgroups in one tissue. As a means to address this question, we provide a detailed separation protocol for the isolation of peroxisomes from both the light (LM-Po) and the heavy (HM-Po) mitochondrial prefraction for their subsequent comparative analysis. Both isolation strategies rely on centrifugation in individually adapted Optiprep gradients. In case of the heavy mitochondrial fraction, free flow electrophoresis is appended as an additional separation step to yield peroxisomes of suf ficient purity. In view of their morphology, peroxisomes isolated from both fractions are surrounded by a continuous single membrane and contain a gray-opaque inner matrix. However, beyond this overall similar appearance, HM-Po exhibit a smaller average diameter, fl oat at lower density, and show a more negative average membrane charge when compared to LM-Po.

3.1870 NADPH oxidase activity in pollen tubes is affected by calcium ions, signaling phospholipids and Rac/Rop GTPases

Potocky, M., Pejhar, P., Gutkowska, M., Jimenez-Quesada, M.J., Potocka, A., de Dios Alche, J., Kost, B. and Zarsky, V.

J. Plant Physiol., ***169***, *1654-1663 (2012)*

Reactive oxygen species (ROS) generated by NADPH oxidase (NOX) are crucial for tip growth of pollen tubes. However, the regulation of NOX activity in pollen tubes remains unknown. Using purified plasma membrane fractions from tobacco and olive pollen and tobacco BY-2 cells, we demonstrate that pollen NOX is activated by calcium ions and low abundant signaling phospholipids, such as phosphatidic acid and phosphatidylinositol 4,5-bisphosphate *in vitro* and *in vivo*. Our data also suggest possible synergism between Ca^{2+} and phospholipid-mediated NOX activation in pollen. Rac/Rop small GTPases are also necessary for normal pollen tube growth and have been proposed to regulate ROS production in root hairs. We show here elevated ROS formation in pollen tubes overexpressing wild-type NtRac5 and constitutively active NtRac5, while overexpression of dominant-negative NtRac5 led to a decrease of ROS in pollen tubes. We also show that PA formed by distinct phospholipases D (PLD) is involved in pathways both upstream and downstream of NOX-mediated ROS generation and identify NtPLDδ as a PLD isoform acting in the ROS response pathway.

3.1871 Single-Transmembrane Domain IGF-II/M6P Receptor: Potential Interaction with G Protein and Its Association with Cholesterol-Rich Membrane Domains

Amritraj, A., Posse de Chaves, E.I., Hawkes, C., MacDonald, R.G. and Kar, S.

Endocrinology, ***153(10)***, *4784-4798 (2012)*

The IGF-II/mannose 6-phosphate (M6P) receptor is a single-transmembrane domain glycoprotein that plays an important role in the intracellular trafficking of lysosomal enzymes and endocytosis-mediated degradation of IGF-II. The receptor may also mediate certain biological effects in response to IGF-II binding by interacting with G proteins. However, the nature of the IGF-II/M6P receptor's interaction with the G protein or with G protein-coupled receptor (GPCR) interacting proteins such as β-arrestin remains unclear. Here we report that [^{125}I]IGF-II receptor binding in the rat hippocampal formation is sensitive to guanosine-5′-[γ-thio]triphosphate, mastoparan, and Mas-7, which are known to interfere with the coupling of the classical GPCR with G protein. Monovalent and divalent cations also influenced [^{125}I]IGF-II receptor binding. The IGF-II/M6P receptor, as observed for several GPCRs, was found to be associated with β-arrestin 2, which exhibits sustained ubiquitination after stimulation with Leu^{27}IGF-II, an IGF-II analog that binds rather selectively to the IGF-II/M6P receptor. Activation of the receptor by Leu^{27}IGF-II induced stimulation of extracellular signal-related kinase 1/2 via a pertussis toxin-dependent pathway. Additionally, we have shown that IGF-II/M6P receptors under normal conditions are associated mostly with detergent-resistant membrane domains, but after stimulation with Leu^{27}IGF-II, are translocated to the detergent-soluble fraction along with a portion of β-arrestin 2. Collectively these results suggest that the IGF-II/M6P receptor may interact either directly or indirectly with G protein as well as β-arrestin 2, and activation of the receptor by an agonist can lead to alteration in its subcellular distribution along with stimulation of an intracellular signaling cascade.

3.1872 Proteolytic Processing Regulates Toll-like Receptor 3 Stability and Endosomal Localization

Qi, R., Singh, D. and Cheng Kao, C.

J. Biol. Chem., ***287(39)***, *32617-32629 (2012)*

Toll-like receptors (TLRs) 3, 7, and 9 are innate immune receptors that recognize nucleic acids from pathogens in endosomes and initiate signaling transductions that lead to cytokine production. Activation of TLR9 for signaling requires proteolytic processing within the ectodomain by endosome-associated proteases. Whether TLR3 requires similar proteolytic processing to become competent for signaling remains unclear. Herein we report that human TLR3 is proteolytically processed to form two fragments in endosomes. Unc93b1 is required for processing by transporting TLR3 through the Golgi complex and to the endosomes. Proteolytic cleavage requires the eight-amino acid Loop1 within leucine-rich repeat 12 of the TLR3 ectodomain. Proteolytic cleavage is not required for TLR3 signaling in response to poly(I:C), although processing could modulate the degree of response toward viral double-stranded RNAs, especially in mouse cells. Both the full-length and cleaved fragments of TLR3 can bind poly(I:C) and are present in endosomes. However, although the full-length TLR3 has a half-life in HEK293T cells of 3 h, the cleaved fragments have half-lives in excess of 7 h. Inhibition of TLR3 cleavage by either treatment with cathepsin

inhibitor or by a mutation in Loop1 decreased the abundance of TLR3 in endosomes targeted for lysosomal degradation.

3.1873 Bax Activation Initiates the Assembly of a Multimeric Catalyst that Facilitates Bax Pore Formation in Mitochondrial Outer Membranes
Kushnavera, Y., Andreyev, A.Y., Kuwana, T. and Newmeyer, D.D.
PloS Biology, ***10(9)***, *e1001394 (2012)*

Bax/Bak-mediated mitochondrial outer membrane permeabilization (MOMP) is essential for "intrinsic" apoptotic cell death. Published studies used synthetic liposomes to reveal an intrinsic pore-forming activity of Bax, but it is unclear how other mitochondrial outer membrane (MOM) proteins might facilitate this function. We carefully analyzed the kinetics of Bax-mediated pore formation in isolated MOMs, with some unexpected results. Native MOMs were more sensitive than liposomes to added Bax, and MOMs displayed a lag phase not observed with liposomes. Heat-labile MOM proteins were required for this enhanced response. A two-tiered mathematical model closely fit the kinetic data: first, Bax activation promotes the assembly of a multimeric complex, which then catalyzes the second reaction, Bax-dependent pore formation. Bax insertion occurred immediately upon Bax addition, prior to the end of the lag phase. Permeabilization kinetics were affected in a reciprocal manner by [cBid] and [Bax], confirming the "hit-and-run" hypothesis of cBid-induced direct Bax activation. Surprisingly, MOMP rate constants were linearly related to [Bax], implying that Bax acts non-cooperatively. Thus, the oligomeric catalyst is distinct from Bax. Moreover, contrary to common assumption, pore formation kinetics depend on Bax monomers, not oligomers. Catalyst formation exhibited a sharp transition in activation energy at ~28°C, suggesting a role for membrane lipid packing. Furthermore, catalyst formation was strongly inhibited by chemical antagonists of the yeast mitochondrial fission protein, Dnm1. However, the mammalian ortholog, Drp1, was undetectable in mitochondrial outer membranes. Moreover, ATP and GTP were dispensable for MOMP. Thus, the data argue that oligomerization of a catalyst protein, distinct from Bax and Drp1, facilitates MOMP, possibly through a membrane-remodeling event.

3.1874 An improved procedure for isolation of functional synaptosomes for the transient generation of cybrids from frozen human brain
Castora, F.J. and Trevino, M.B.
FASEB J., ***26***, *586.4 (2012)*

Cybrids are cell lines derived from the fusion of mtDNA-depleted cells with cytoplasts or enucleated cells. To date, there are no reports of cybrid fusions from frozen human tissue. In this study, we developed a new method of synaptosome isolation, the "Frozen Brain Synaptosomes" or FBS method. Synaptosomes were isolated from fresh mouse, frozen mouse and frozen human brain by centrifugation in an iodixanol gradient. The effectiveness of our FBS method was compared with current synaptosome isolation procedures that have been utilized to make cybrid cell lines from fresh tissue. The FBS method yielded the most structurally intact synaptosome preparation from frozen tissue as judged by electron microscopy. We assessed cytochrome c oxidase and citrate synthase activity, phospho-synapsin-1 and total ATP levels. The FBS method yielded the greatest enrichment of intact synaptosomes, high protein, mitochondrial enzyme activity and ATP levels, and functional synaptosomes as defined by their indirect response to a phosphatase inhibitor okadaic acid (OKA). We expected that the synaptosomes isolated from frozen postmortem brain would be sufficient in quantity and quality to generate cybrid cells. From ten fusion experiments, we concluded that two putative cybrid cell lines contained the exogenous synaptosomal mitochondria based upon mtDNA sequence analysis. Research support was from the Virginia Center on Aging.

3.1875 Overexpression of the Coq8 Kinase in Saccharomyces cerevisiae coq Null Mutants Allows for Accumulation of Diagnostic Intermediates of the Coenzyme Q6 Biosynthetic Pathway
Xie, L.X., Ozeir, M., Tang, J.Y., Chen, J.Y., Jaquinod, S-K., Fonteecave, M., Clarke, C.F. ansd Pierrel, F.
J. Biol. Chem., ***287(28)***, *23571-23581 (2012)*

Most of the Coq proteins involved in coenzyme Q (ubiquinone or Q) biosynthesis are interdependent within a multiprotein complex in the yeast *Saccharomyces cerevisiae*. Lack of only one Coq polypeptide, as in Δ*coq* strains, results in the degradation of several Coq proteins. Consequently, Δ*coq* strains accumulate the same early intermediate of the Q_6 biosynthetic pathway; this intermediate is therefore not informative about the deficient biosynthetic step in a particular Δ*coq* strain. In this work, we report that the overexpression of the protein Coq8 in Δ*coq* strains restores steady state levels of the unstable Coq proteins.

Coq8 has been proposed to be a kinase, and we provide evidence that the kinase activity is essential for the stabilizing effect of Coq8 in the Δ*coq* strains. This stabilization results in the accumulation of several novel Q_6 biosynthetic intermediates. These Q intermediates identify chemical steps impaired in cells lacking Coq4 and Coq9 polypeptides, for which no function has been established to date. Several of the new intermediates contain a C4-amine and provide information on the deamination reaction that takes place when *para*-aminobenzoic acid is used as a ring precursor of Q_6. Finally, we used synthetic analogues of 4-hydroxybenzoic acid to bypass deficient biosynthetic steps, and we show here that 2,4-dihydroxybenzoic acid is able to restore Q_6 biosynthesis and respiratory growth in a Δ*coq7* strain overexpressing Coq8. The overexpression of Coq8 and the use of 4-hydroxybenzoic acid analogues represent innovative tools to elucidate the Q biosynthetic pathway.

3.1876 Specific and Nonspecific Regulation of GPCR Function by Cholesterol
Gimpl, G. and Gehrig-Burger, K.
Cholesterol Regulation of Ion Channels and Receptors, 205-230 (2012)

Cholesterol regulates the physical state of the phospholipid bilayer and is crucially involved in the formation of membrane microdomains. In view of the abundance of cholesterol in the plasma membrane of eukaryotic cells, any integral membrane protein should always be in close molecular contact with cholesterol. This is particularly true for the heptahelical G-protein-coupled receptors (GPCRs) that form the largest receptor superfamily. Owing to their seven transmembrane helices, large parts of these proteins are embedded in the cholesterol-rich plasma membrane bilayer. Some GPCRs have been shown to be functionally dependent on cholesterol (Table 10.1; Burger et al., 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010). It is difficult to clarify whether such cholesterol dependence is based on direct interaction with cholesterol or on indirect effects caused by the influence of cholesterol on the biophysical state of the membrane, for example, changes in the membrane fluidity. The following questions (Sections 10.2–10.8) might be addressed in order to prove or to provide evidence whether a candidate GPCR is functionally dependent on cholesterol, and, if so, to what extent this is based on direct cholesterol–receptor interaction.

3.1877 Rotaviral Enterotoxin Nonstructural Protein 4 Targets Mitochondria for Activation of Apoptosis during Infection
Bhowmick, R., Chandra Halder, U., Chattopadhyay, S., Chanda, S., Nandi, S., Bagchi, P., Nayak, M.K., Chakrabarti, O., Kobayashi, N. and Chawla-Sarkar, M.
*J. Biol. Chem., **287(42)**, 35004-35020 (2012)*

Viruses have evolved to encode multifunctional proteins to control the intricate cellular signaling pathways by using very few viral proteins. Rotavirus is known to express six nonstructural and six structural proteins. Among them, NSP4 is the enterotoxin, known to disrupt cellular Ca^{2+} homeostasis by translocating to endoplasmic reticulum. In this study, we have observed translocation of NSP4 to mitochondria resulting in dissipation of mitochondrial membrane potential during virus infection and NSP4 overexpression. Furthermore, transfection of the N- and C-terminal truncated NSP4 mutants followed by analyzing NSP4 localization by immunofluorescence microscopy identified the 61–83-amino acid region as the shortest mitochondrial targeting signal. NSP4 exerts its proapoptotic effect by interacting with mitochondrial proteins adenine nucleotide translocator and voltage-dependent anion channel, resulting in dissipation of mitochondrial potential, release of cytochrome *c* from mitochondria, and caspase activation. During early infection, apoptosis activation by NSP4 was inhibited by the activation of cellular survival pathways (PI3K/AKT), because PI3K inhibitor results in early induction of apoptosis. However, in the presence of both PI3K inhibitor and NSP4 siRNA, apoptosis was delayed suggesting that the early apoptotic signal is initiated by NSP4 expression. This proapoptotic function of NSP4 is balanced by another virus-encoded protein, NSP1, which is implicated in PI3K/AKT activation because overexpression of both NSP4 and NSP1 in cells resulted in reduced apoptosis compared with only NSP4-expressing cells. Overall, this study reports on the mechanism by which enterotoxin NSP4 exerts cytotoxicity and the mechanism by which virus counteracts it at the early stage for efficient infection.

3.1878 Compromised Mitochondrial Fatty Acid Synthesis in Transgenic Mice Results in Defective Protein Lipoylation and Energy Disequilibrium
Smith, S. et al
*Plos One, **7(10)**, e47196 (2012)*

A mouse model with compromised mitochondrial fatty acid synthesis has been engineered in order to assess the role of this pathway in mitochondrial function and overall health. Reduction in the expression of mitochondrial malonyl CoA-acyl carrier protein transacylase, a key enzyme in the pathway encoded by the nuclear *Mcat* gene, was achieved to varying extents in all examined tissues employing tamoxifen-inducible *Cre-lox* technology. Although affected mice consumed more food than control animals, they failed to gain weight, were less physically active, suffered from loss of white adipose tissue, reduced muscle strength, kyphosis, alopecia, hypothermia and shortened lifespan. The Mcat-deficient phenotype is attributed primarily to reduced synthesis, in several tissues, of the octanoyl precursors required for the posttranslational lipoylation of pyruvate and α-ketoglutarate dehydrogenase complexes, resulting in diminished capacity of the citric acid cycle and disruption of energy metabolism. The presence of an alternative lipoylation pathway that utilizes exogenous free lipoate appears restricted to liver and alone is insufficient for preservation of normal energy metabolism. Thus, de novo synthesis of precursors for the protein lipoylation pathway plays a vital role in maintenance of mitochondrial function and overall vigor.

3.1879 MS-275 sensitizes osteosarcoma cells to Fas ligand-induced cell death by increasing the localization of Fas in membrane lipid rafts

Rao-Lindahl, K., Zhou, Z. and Kleinermann, E.S.
*Cell Death and Disease, **3**, e369 (2012)*

Fas expression is inversely correlated with the metastatic potential of osteosarcoma (OS) cells to the lungs. Fas^+ cells are rapidly eliminated when they enter the lungs via their interaction with constitutive Fas ligand (FasL) on the lung epithelium, whereas Fas^- OS cells escape this FasL-induced apoptosis and survive in the lung microenvironment. Upregulation of Fas expression in established OS lung metastases results in tumor regression. Here, we demonstrate that treatment of Fas^- OS cells with the histone deacetylase inhibitor MS-275 results in the upregulation of Fas mRNA and sensitizes these cells to FasL-induced apoptosis. However, flow cytometry analysis revealed that Fas cell surface protein expression was not significantly increased. Rather, we observed increased levels of Fas within the membrane lipid rafts, as demonstrated by an increase in Fas expression in detergent-insoluble lipid raft fractions and colocalization with $GM1^+$ lipid rafts. We had previously shown that MS-275 treatment inhibited expression of the anti-apoptotic cellular FLICE-inhibitory protein (c-FLIP). Here, we demonstrated that transfection of cells with short hairpin RNA to c-FLIP also resulted in the localization of Fas to lipid rafts. Overall, our studies indicate that MS-275 sensitizes OS cells to FasL by upregulating the expression of Fas in membrane lipid rafts, which correlates with the c-FLIP-dependent distribution of Fas to lipid rafts.

3.1880 HDL and ApoA-I inhibit antigen presentation-mediated T cell activation by disrupting lipid rafts in antigen presenting cells

Wang, S-h., Yuan, S-g., Peng, D-q. and Zhao, S-p.
*Atherosclerosis, **225**, 105-114 (2012)*

Objective
Depletion of cholesterol by methyl-β-cyclodextrin (MCD) on peptide-loaded antigen presenting cells (APCs) inhibits antigen presentation and T cell activation. However, whether membrane cholesterol efflux induced by high-density lipoprotein (HDL) and apolipoprotein A-I (apoA-I) also results in inhibition of antigen presentation and T cell activation is still unknown.
Methods and results
Various types of APCs, including B cells, macrophages and dendritic cells (DCs), were first loaded with antigen, then incubated with HDL and apoA-I to decrease cellular membrane cholesterol content. After being treated with HDL and apoA-I, APCs demonstrated decreased potential to activate T cells, and this decrease correlated with an increase in cholesterol efflux from APCs. Cholesterol repletion reversed the inhibitory effects of HDL and apoA-I, demonstrating that the observed reduction in T cell proliferation is mediated through cholesterol. Furthermore, lipid raft analysis showed that HDL and apoA-I reduced cholesterol and major histocompatibility (MHC) class II protein content in lipid rafts, suggesting that cholesterol efflux from APCs to HDL and apoA-I inhibits antigen presentation and T cell activation by reducing lipid rafts assembly in APCs.
Conclusion
HDL and apoA-I inhibit the capacity of APCs to stimulate T cell activation, and this inhibition can be attributed to cholesterol efflux and the ensuing disruption of plasma membrane lipid rafts in APCs. Overall, these findings suggest that cholesterol efflux mediated by HDL and apoA-I may serve to link immunity and cardioprotection.

3.1881 Characterizing Synaptic Vesicle Proteins Using Synaptosomal Fractions and Cultured Hippocampal Neurons
DiGiovanni, J., Sun, T. and Sheng, Z-H.
Current Protocols in Neuroscience, Suppl. 59, 2.7.1-2.7.2 (2012)

Cloning and characterization of synaptic vesicle proteins and their binding counterparts on the presynaptic plasma membrane have greatly advanced our understanding of the molecular mechanisms involved in the synaptic vesicle cycle and neurotransmitter release. This unit discusses multidisciplinary approaches to characterize proteins from synaptosome-enriched subcellular fractions and localize them within cultured neurons. The first approach regroups methods used to isolate synaptic vesicles from rat brain synaptosomal preparations, allowing for specific biochemical investigation of synaptic vesicle proteins. The second is a detailed procedure for pre-embedding immunogold staining and electron microscopic observation, which permits the morphological identification of proteins in individual vesicles at intact synapses. Additionally, this chapter proposes methods for light microscopic examination of hippocampal neurons. It includes procedures for embryonic and postnatal hippocampal neuron culture and describes an immunocytochemical staining protocol used to investigate synaptic vesicle protein localization with respect to other proteins or subcellular structures.

3.1882 Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems
Ilina, P., Hyvonen, Z., Saura, M., Sandvig, K., Yliperttula, M. and Ruponen, M.
*J. Controlled Release, **161**, 385-395 (2012)*

Detailed understanding of the uptake mechanisms and intracellular processing of nonviral gene delivery systems will allow design of more effective carriers. This work gets insight into the intracellular kinetics of pDNA delivered by polyethyleneimine (PEI), cationic lipid DOTAP and calcium phosphate (CaP) precipitates. Amount of cell- and nuclear-associated pDNA was quantified by qRT-PCR at multiple time points after transfection. Moreover, the impact of specific endocytic pathways on the cell entry and intracellular kinetics of pDNA was studied by inhibition (blockage) of either clathrin- or dynamin-mediated endocytosis by using both genetically manipulated cell lines and chemical inhibitors of endocytosis. Quantitative analysis of defined kinetic parameters revealed that neither cellular nor nuclear uptake of pDNA correlated with transgene expression, emphasizing the importance of the post-nuclear processes in overall transfection efficacy. Changes in transgene expression observed upon blockage of endocytosis was carrier dependent and correlated relatively well with the changes at the cellular and nuclear uptake levels but not with the amount of cell-associated pDNA. Due to low specificity of chemical inhibitors and activation of alternative endocytosis pathways after genetic blockage of endocytosis neither of these methods is optimal for studying the role of endocytosis. Therefore, one should be careful when interpreting the obtained results from such studies and not to trust the data obtained only from one method.

3.1883 Identification of Proteins Associated with the Pseudomonas aeruginosa Biofilm Extracellular Matrix
Toyofuku, M., Roschitzki, B., Riedel, K. and Eberl, L.
*J. Proteome Res., **11**, 4906-4915 (2012)*

Biofilms are surface-associated bacteria that are embedded in a matrix of self-produced polymeric substances (EPSs). The EPS is composed of nucleic acids, polysaccharides, lipids, and proteins. While polysaccharide components have been well studied, the protein content of the matrix is largely unknown. Here we conducted a comprehensive proteomic study to identify proteins associated with the biofilm matrix of *Pseudomonas aeruginosa* PAO1 (the matrix proteome). This analysis revealed that approximately 30% of the identified matrix proteins were outer membrane proteins, which are also typically found in outer membrane vesicles (OMVs). Electron microscopic inspection confirmed the presence of large amounts of OMVs within the biofilm matrix, supporting previous notions that OMVs are abundant constituents of *P. aeruginosa* biofilms. Our results demonstrate that while some proteins associated with the *P. aeruginosa* matrix are derived from secreted proteins and lysed cells, the large majority of the matrix proteins originate from OMVs. Furthermore, we demonstrate that the protein content of planktonic and biofilm OMVs is surprisingly different and may reflect the different physiological states of planktonic and sessile cells.

3.1884 Pharmacological chaperones for human α-N-acetylgalactosaminidase
Clark, N.E., Metcalf, M.C., Best, D., Fleet, G.W.J. and Garman, S.C.

*PNAS, **109(43)**, 17400-17405 (2012)*

Schindler/Kanzaki disease is an inherited metabolic disease with no current treatment options. This neurologic disease results from a defect in the lysosomal α-*N*-acetylgalactosaminidase (α-NAGAL) enzyme. In this report, we show evidence that the iminosugar DGJNAc can inhibit, stabilize, and chaperone human α-NAGAL both in vitro and in vivo. We demonstrate that a related iminosugar DGJ (currently in phase III clinical trials for another metabolic disorder, Fabry disease) can also chaperone human α-NAGAL in Schindler/Kanzaki disease. The 1.4- and 1.5-Å crystal structures of human α-NAGAL complexes reveal the different binding modes of iminosugars compared with glycosides. We show how differences in two functional groups result in >9 kcal/mol of additional binding energy and explain the molecular interactions responsible for the unexpectedly high affinity of the pharmacological chaperones. These results open two avenues for treatment of Schindler/Kanzaki disease and elucidate the atomic basis for pharmacological chaperoning in the entire family of lysosomal storage diseases.

3.1885 Protein abundance of urea transporters and aquaporin 2 change differently in nephrotic pair-fed vs. non-pair-fed rats

Matar, R.N.M., Malik, B., Wang, X.H., Martin, C.F., Eaton, D.C., Sands, J.M. and Klein, J.D.
*Am. J. Physiol. Renal Physiol., **302(12)**, F1545-F1553 (2012)*

Salt and water retention is a hallmark of nephrotic syndrome (NS). In this study, we test for changes in the abundance of urea transporters, aquaporin 2 (AQP2), Na-K-2Cl cotransporter 2 (NKCC2), and Na-Cl cotransporter (NCC), in non-pair-fed and pair-fed nephrotic animals. Doxorubicin-injected male Sprague-Dawley rats (n = 10) were followed in metabolism cages. Urinary excretion of protein, sodium, and urea was measured periodically. Kidney inner medulla (IM), outer medulla, and cortex tissue samples were dissected and analyzed for mRNA and protein abundances. At 3 wk, all doxorubicin-treated rats developed features of NS, with a ninefold increase in urine protein excretion (from 144 ± 21 to 1,107 ± 165 mg/day; $P < 0.001$) and reduced urinary sodium excretion (from 0.17 to 0.12 meq/day; $P < 0.001$). Urine osmolalities were reduced in the nephrotic animals (1,057 ± 37, treatment vs. 1,754 ± 131, control). Unlike animals fed ad libitum, UT-A1 protein abundance was unchanged in nephrotic pair-fed rats. Glycosylated AQP2 was reduced in the IM base of both nephrotic groups. Abundances of NKCC2 and NCC were consistently reduced (71 ± 7 and 33 ± 13%, respectively) in both nephrotic pair-fed animals and animals fed ad libitum. In pair-fed nephrotic rats, we observed an increase in the cleaved form of membrane-bound γ-epithelial sodium channel (ENaC). However, α- and β-ENaC subunits were unaltered. NKCC2 and AQP2 mRNA levels were similar in treated vs. control rats. We conclude that dietary protein intake affects the response of medullary transport proteins to NS.

3.1886 Lipid rafts, microdomain heterogeneity and inter-organelle contacts: Impacts on membrane preparation for proteomic studies

Minogue, S. and Waugh, M.G.
Biol. Cell, 104(10), 618-627 (2012)

In recent years, there has been considerable interest in mapping the protein content of isolated organelles using mass spectrometry. However, many subcellular compartments are highly dynamic with diverse and intricate architectures that are not always preserved during membrane isolation procedures. Furthermore, lateral heterogeneities in intra-membrane lipid and protein concentrations underlie the formation of membrane microdomains, trafficking vesicles and inter-membrane contacts. These complexities in membrane organisation have important consequences for the design of membrane preparation strategies and test the very concept of organelle purity. We illustrate how some of these biological considerations are relevant to membrane preparation and assess the numerous potential pitfalls in attempting to purify organelles from mammalian cells.

3.1887 TPC Proteins Are Phosphoinositide- Activated Sodium-Selective Ion Channels in Endosomes and Lysosomes

Wang, X., Zhang, X., Dong, X-p., Samie, M., Li, X., Cheng, X., Goschka, A., Shen, D., Zhou, Y., Harlow, J., Zhu, M.X., Clapham, D.E., Ren, D. and Xu, H.
*Cell, **151(2)**, 372-383 (2012)*

Mammalian two-pore channel proteins (TPC1, TPC2; *TPCN1*, *TPCN2*) encode ion channels in intracellular endosomes and lysosomes and were proposed to mediate endolysosomal calcium release triggered by the second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP). By directly

recording TPCs in endolysosomes from wild-type and TPC double-knockout mice, here we show that, in contrast to previous conclusions, TPCs are in fact sodium-selective channels activated by $PI(3,5)P_2$ and are not activated by NAADP. Moreover, the primary endolysosomal ion is Na^+, not K^+, as had been previously assumed. These findings suggest that the organellar membrane potential may undergo large regulatory changes and may explain the specificity of $PI(3,5)P_2$ in regulating the fusogenic potential of intracellular organelles.

3.1888 Stabilization of Kv1.5 channel protein by bepridil through its action as a chemical chaperone
Suzuki, S., Kurata, Y., Li, P., Notsu, T., Hasegawa, A., Ikeda, N., kato, M., Miake, J., Sakata, S., Shiota, G., Yoshida, A., Ninomiya, H., Higaki, K., Yamamoto, K., Shirayoshi, Y. and Hisatome, I.
Eur. J. Pharmacol., ***696****, 28-34 (2012)*

While bepridil has been reported to alter the stability of ion channel proteins, the precise mechanism of action remains unclear. We examined the effect of bepridil on the stability of Kv1.5 channel proteins expressed in COS7 cells. Bepridil at 0.3–30 μM increased the protein level of Kv1.5 channels in a concentration-dependent manner. Chase experiments showed that bepridil delayed the degradation process of Kv1.5 channel proteins in the same manner as a proteasomal inhibitor, MG132, did. Bepridil increased the immunofluorescent signal of Kv1.5 channel proteins in the endoplasmic reticulum (ER) and Golgi apparatus and on the cell surface. The cell fraction experiment also showed bepridil-induced increases in Kv1.5 in the ER, Golgi apparatus, and the cell membrane. Bepridil at a lower concentration of 1 μM had no effect on the proteasome activity in vitro. A blocker of the ultrarapid delayed-rectifier K^+ channel current, 4-aminopyridine (4AP), abolished bepridil-induced increases in Kv1.5. Kv1.5-medicated membrane currents measured as 4AP-sensitive currents were increased by bepridil. Taken together, we conclude that bepridil stabilizes Kv1.5 proteins at the ER through an action as a chemical chaperone, thereby increasing the density of Kv1.5 channels in the cell membrane.

3.1889 Review on recent advances in the analysis of isolated organelles
Satori, C.P., Kostal, V. and Arriaga, E.A.
Analytica Chimica Acta, ***753****, 8-18 (2012)*

The analysis of isolated organelles is one of the pillars of modern bioanalytical chemistry. This review describes recent developments on the isolation and characterization of isolated organelles both from living organisms and cell cultures. Salient reports on methods to release organelles focused on reproducibility and yield, membrane isolation, and integrated devices for organelle release. New developments on organelle fractionation after their isolation were on the topics of centrifugation, immunocapture, free flow electrophoresis, flow field-flow fractionation, fluorescence activated organelle sorting, laser capture microdissection, and dielectrophoresis. New concepts on characterization of isolated organelles included atomic force microscopy, optical tweezers combined with Raman spectroscopy, organelle sensors, flow cytometry, capillary electrophoresis, and microfluidic devices.

3.1890 TRAIL-activated EGFR by Cbl-b-regulated EGFR redistribution in lipid rafts antagonises TRAIL-induced apoptosis in gastric cancer cells
Xu, L., Zhang, Y., Liu, J., Qu, J., Hu, X., Zhang, F., Zheng, H., Qu, X. and Liu, Y.
Eur. J. Cancer, ***48****, 3288-3299 (2012)*

Most gastric cancer cells are resistant to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Since TRAIL resistance is associated with lipid rafts, in which both death receptors and epidermal growth factor receptors (EGFR) are enriched, our aim is to identify how lipid raft-regulated receptor redistribution influences the sensitivity of TRAIL in gastric cancer cells. In TRAIL-resistant gastric cancer cells, TRAIL did not induce effective death-inducing signalling complex (DISC) formation in lipid rafts, accompanied with EGFR translocation into lipid rafts, and activation of EGFR pathway. Knockdown of casitas B-lineage lymphoma-b (Cbl-b) enhanced TRAIL-induced apoptosis by promoting DISC formation in lipid rafts. However, knockdown of Cbl-b also enhanced EGFR translocation into lipid rafts and EGFR pathway activation induced by TRAIL. Either using inhibitors of EGFR or depletion of EGFR with small interfering RNA (siRNA) prevented EGFR pathway activation, and thus increased TRAIL-induced apoptosis, especially in Cbl-b knockdown clones. Taken together, TRAIL-induced EGFR activation through Cbl-b-regulated EGFR redistribution in lipid rafts antagonised TRAIL-induced apoptosis. The contribution of DISC formation and the inhibition of EGFR signal triggered in lipid rafts are both essential for increasing the sensitivity of gastric cancer cells to TRAIL.

3.1891 Localisation of a family of complex-forming β-barrels in the T. vaginalis hydrogenosomal membrane
Kay, C., Lawler, K., Self, T.J., Dyall, S.D. and Kerr, I.D.
FEBS Lett., ***586***, *4038-4045 (2012)*

Crucial to organellogenesis was the development of membrane translocases responsible for delivering proteins to new cellular compartments. This investigation examines the *Trichomonas vaginalis* hydrogenosome, a mitochondrially derived organelle. We identify an expanded family of putative β-barrel proteins (THOM A–I) comprising nine related sequences. Sub-cellular localisation by immunofluorescence and biochemical fractionation is consistent with THOMs being localised to the hydrogenosomal membrane. Native gel electrophoresis and chemical cross-linking support the ability of THOM proteins to be components of membrane-bound oligomeric protein complexes, consistent with a role in protein translocation.

3.1892 Vaspin Is an Adipokine Ameliorating ER Stress in Obesity as a Ligand for Cell-Surface GRP78/MTJ-1 Complex
Nakatsuka, A. et al
Diabetes, ***61***, *2823-2832 (2012)*

It is unknown whether adipokines derived from adipose tissues modulate endoplasmic reticulum (ER) stress induced in obesity. Here, we show that visceral adipose tissue–derived serine protease inhibitor (vaspin) binds to cell-surface 78-kDa glucose-regulated protein (GRP78), which is recruited from ER to plasma membrane under ER stress. Vaspin transgenic mice were protected from diet-induced obesity, glucose intolerance, and hepatic steatosis, while vaspin-deficient mice developed glucose intolerance associated with upregulation of ER stress markers. With tandem affinity tag purification using HepG2 cells, we identified GRP78 as an interacting molecule. The complex formation of vaspin, GRP78, and murine tumor cell DnaJ-like protein 1 (MTJ-1) (DnaJ homolog, subfamily C, member 1) on plasma membrane was confirmed by cell-surface labeling with biotin and immunoprecipitation in liver tissues and H-4-II-E-C3 cells. The addition of recombinant human vaspin in the cultured H-4-II-E-C3 cells also increased the phosphorylation of Akt and AMP-activated protein kinase (AMPK) in a dose-dependent manner, and anti-GRP78 antibodies completely abrogated the vaspin-induced upregulation of pAkt and pAMPK. Vaspin is a novel ligand for cell-surface GRP78/MTJ-1 complex, and its subsequent signals exert beneficial effects on ER stress–induced metabolic dysfunctions.

3.1893 Down-regulation of connexin43 expression reveals the involvement of caveolin-1 containing lipid rafts in human U251 glioblastoma cell invasion
Strale. P-O., Clarhaut, J., Lamiche, C., Cronier, L., Mesnil, M. and Defamie, N.
Mol. Carcinogenesis, ***51(11)***, *845-860 (2012)*

Glioblastoma cells are characterized by high proliferation and invasive capacities. Tumor development has been associated with a decrease of gap-junctional intercellular communication, but the concrete involvement of gap junction proteins, connexins, remains elusive since they are also suspected to promote cell invasion. In order to better understand how connexins control the glioma cell phenotype, we studied the consequences of inhibiting the intrinsic expression of the major astrocytic connexin, Connexin43, in human U251 glioblastoma cells by the shRNA strategy. The induced down-regulation of Cx43 expression has various effects on the U251 cells such as increased clonogenicity, angiogenesis and decreased adhesion on specific extracellular matrix proteins. We demonstrate that the invasion capacity measured in vitro and ex vivo correlates with Cx43 expression level. For the first time in a cancer cell context, our work demonstrates that Cx43 cofractionates, colocalizes and coimmunoprecipitates with a lipid raft marker, caveolin-1 and that this interaction is inversely correlated to the level of Cx43. This localization of Cx43 in these lipid raft microdomains regulates both homo- and heterocellular gap junctional communications (respectively between U251 cells, or between U251 cells and astrocytes). Moreover, the adhesive and invasive capacities are not dependent, in our model, on Cav-1 expression level. Our results tend to show that heterocellular gap junctional communication between cancer and stroma cells may affect the behavior of the tumor cells. Altogether, our data demonstrate that Cx43 controls the tumor phenotype of glioblastoma U251 cells and in particular, invasion capacity, through its localization in lipid rafts containing Cav-1.

3.1894 Cryptococcus neoformans-Derived Microvesicles Enhance the Pathogenesis of Fungal Brain Infection
Huang, S-H., Wu, C-H., Chang, Y.C., Kwon-Chung, K.J., Brown, R.J. and Jong, A.

*PloS One, **7(11)**, e48570 (2012)*

Cryptococcal meningoencephalitis is the most common fungal disease in the central nervous system. The mechanisms by which *Cryptococcus neoformans* invades the brain are largely unknown. In this study, we found that *C. neoformans*-derived microvesicles (CnMVs) can enhance the traversal of the blood-brain barrier (BBB) by *C. neoformans in vitro*. The immunofluorescence imaging demonstrates that CnMVs can fuse with human brain microvascular endothelial cells (HBMECs), the constituents of the BBB. This activity is presumably due to the ability of the CnMVs to activate HBMEC membrane rafts and induce cell fusogenic activity. CnMVs also enhanced *C. neoformans* infection of the brain, found in both infected brains and cerebrospinal fluid. In infected mouse brains, CnMVs are distributed inside and around *C. neoformans*-induced cystic lesions. GFAP (glial fibrillary acidic protein)-positive astrocytes were found surrounding the cystic lesions, overlapping with the 14-3-3-GFP (14-3-3-green fluorescence protein fusion) signals. Substantial changes could be observed in areas that have a high density of CnMV staining. This is the first demonstration that *C. neoformans*-derived microvesicles can facilitate cryptococcal traversal across the BBB and accumulate at lesion sites of *C. neoformans*-infected brains. Results of this study suggested that CnMVs play an important role in the pathogenesis of cryptococcal meningoencephalitis.

3.1895 STARD4 knockdown in HepG2 cells disrupts cholesterol trafficking associated with the plasma membrane, ER, and ERC
Garbarino, J., Pan, M., Chin, H.F., Lund, F.W., Maxfield, F.R. and Breslow, J.L.
*J. Lipid Res., **53**, 2716-2725 (2012)*

STARD4, a member of the evolutionarily conserved START gene family, has been implicated in the nonvesicular intracellular transport of cholesterol. However, the direction of transport and the membranes with which this protein interacts are not clear. We present studies of STARD4 function using small hairpin RNA knockdown technology to reduce STARD4 expression in HepG2 cells. In a cholesterol-poor environment, we found that a reduction in STARD4 expression leads to retention of cholesterol at the plasma membrane, reduction of endoplasmic reticulum-associated cholesterol, and decreased ACAT synthesized cholesteryl esters. Furthermore, D4 KD cells exhibited a reduced rate of sterol transport to the endocytic recycling compartment after cholesterol repletion. Although these cells displayed normal endocytic trafficking in cholesterol-poor and replete conditions, cell surface low density lipoprotein receptor (LDLR) levels were increased and decreased, respectively. We also observed a decrease in NPC1 protein expression, suggesting the induction of compensatory pathways to maintain cholesterol balance. These data indicate a role for STARD4 in nonvesicular transport of cholesterol from the plasma membrane and the endocytic recycling compartment to the endoplasmic reticulum and perhaps other intracellular compartments as well.

3.1896 Update on Methods and Techniques to Study Endocytosis in Plants
Samajova, O., Takac, T., von Wangenheim, D., Stelzer, E. and Samaj, J.
Endocytosis in Plants, 1-36 (2012)

The growing interest in the investigation of endocytosis, vesicular transport routes, and corresponding regulatory mechanisms resulted in the exploitation of cell biological, genetic, biochemical, and proteomic approaches. Methods and techniques such as site-directed and T-DNA insertional mutagenesis, RNAi, classical inhibitor treatments, and recombinant GFP technology combined with confocal laser scanning microscopy (CLSM) and electron and immune-electron microscopy were routinely employed for investigation of endocytosis in plant cells. However, new approaches such as high-throughput confocal microscopy screens on mutants and proteomic analyses on isolated vesicular compartments and root cells treated with vesicular trafficking inhibitors (both focused on the identification of new endosomal proteins), together with chemical genomics and advanced microscopy approaches such as Förster resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), light sheet-based fluorescence microscopy, and super-resolution microscopy provided a significant amount of new data and these new methods appear as extremely promising tools in this field.

3.1897 Sterol C22-Desaturase and Its Biological Roles
Ohta, D. and Mizutani, M.
Isoprenoid Synthesis in Plant and M icroorganism, 381-391 (2012)

The C22-unsaturated sterols are primarily found in fungi and plants. The C22-desaturation reaction is catalyzed by independent cytochrome P450 family proteins, CYP61 in fungi, and CYP710 in plants. We describe our extensive characterization studies of plant CYP710 family proteins and discuss possible evolutional relationships of C22-desaturation reactions among eukaryotic organisms. We also discuss possible research directions toward understanding physiological implications of sterols in unidentified brassinosteroid-independent growth/developmental processes.

3.1898 Proteomic Characterization of Phagosomal Membrane Microdomains During Phagolysosome Biogenesis and Evolution

Goyette, G., Boulais, J., Carruthers, N.J., Landry, C.R., Justras, I., Duclos, S., Dermine, J-F., Michnick, S.W., LaBoissiere, S., Lajoie, G., Barrereiro, L., Thibault, P. and Desjardin, M.
*Mol. Cell. Proteomics, **11**, 1365-1377 (2012)*

After their formation at the cell surface, phagosomes become fully functional through a complex maturation process involving sequential interactions with various intracellular organelles. In the last decade, series of data indicated that some of the phagosome functional properties occur in specialized membrane microdomains. The molecules associated with membrane microdomains, as well as the organization of these structures during phagolysosome biogenesis are largely unknown. In this study, we combined proteomics and bioinformatics analyses to characterize the dynamic association of proteins to maturing phagosomes. Our data indicate that groups of proteins shuffle from detergent-soluble to detergent-resistant membrane microdomains during maturation, supporting a model in which the modulation of the phagosome functional properties involves an important reorganization of the phagosome proteome by the coordinated spatial segregation of proteins.

3.1899 Genetic Depletion of Complement Receptors CD21/35 Prevents Terminal Prion Disease in a Mouse Model of Chronic Wasting Disease

Brady, M., Ferguson, A., Johnson, T., Bender, H., Meyerett-reid, C., Pulford, B., von Teichman, A., Seelig, D., Weis, J.H., Telling, G.C., Aguzzi, A. and Zabel, M.D.
J. Immunol., 189, 4520-4527 (2012)

The complement system has been shown to facilitate peripheral prion pathogenesis. Mice lacking complement receptors CD21/35 partially resist terminal prion disease when infected i.p. with mouse-adapted scrapie prions. Chronic wasting disease (CWD) is an emerging prion disease of captive and free-ranging cervid populations that, similar to scrapie, has been shown to involve the immune system, which probably contributes to their relatively facile horizontal and environmental transmission. In this study, we show that mice overexpressing the cervid prion protein and susceptible to CWD (Tg(cerPrP)5037 mice) but lack CD21/35 expression completely resist clinical CWD upon peripheral infection. CD21/35-deficient Tg5037 mice exhibit greatly impaired splenic prion accumulation and replication throughout disease, similar to CD21/35-deficient murine prion protein mice infected with mouse scrapie. TgA5037;CD21/35−/− mice exhibited little or no neuropathology and deposition of misfolded, protease-resistant prion protein associated with CWD. CD21/35 translocate to lipid rafts and mediates a strong germinal center response to prion infection that we propose provides the optimal environment for prion accumulation and replication. We further propose a potential role for CD21/35 in selecting prion quasi-species present in prion strains that may exhibit differential zoonotic potential compared with the parental strains.

3.1900 SMAD versus Non-SMAD Signaling Is Determined by Lateral Mobility of Bone Morphogenetic Protein (BMP) Receptors

Guzman, A., Zelman-Femiak, M., Boergermann, J.H., Paschowsky, S., Kreuzaler, P.A., Fratzl, P., Harms, G.S. and Knaus, P.
*J. Biol. Chem., **287(47)**, 39492-39504 (2012)*

Bone (or body) morphogenetic proteins (BMPs) belong to the TGFβ superfamily and are crucial for embryonic patterning and organogenesis as well as for adult tissue homeostasis and repair. Activation of BMP receptors by their ligands leads to induction of several signaling cascades. Using fluorescence recovery after photobleaching, FRET, and single particle tracking microscopy, we demonstrate that BMP receptor type I and II (BMPRI and BMPRII) have distinct lateral mobility properties within the plasma membrane, which is mandatory for their involvement in different signaling pathways. Before ligand binding, BMPRI and a subpopulation of BMPRII exhibit confined motion, reflecting preassembled heteromeric receptor complexes. A second free diffusing BMPRII population only becomes restricted after

ligand addition. This paper visualizes time-resolved BMP receptor complex formation and demonstrates that the lateral mobility of BMPRI has a major impact in stabilizing heteromeric BMPRI-BMPRII receptor complexes to differentially stimulate SMAD *versus* non-SMAD signaling.

3.1901 Tollip, an Intracellular Trafficking Protein, Is a Novel Modulator of the Transforming Growth Factor-β Signaling Pathway
Zhu, L., Wang, L., Luo, X., Zhang, Y., Ding, Q., Jiang, X., Wang, X., Pan, Y. and Chen, Y.
J. Biol. Chem., ***287(47)***, *39653-39663 (2012)*

Upon activation, TGF-β type I receptor (TβRI) undergoes active ubiquitination via recruitment of E3 ligases to the receptor complex by Smad7. However, how ubiquitination of TβRI is coupled to intracellular trafficking, and protein degradation remains unclear. We report here that Tollip, an adaptor protein that contains both ubiquitin-associated domains and endosome-targeting domain, plays an important role in modulating trafficking and degradation of TβRI. Tollip was previously demonstrated to possess a functional role in modulating the signaling of interleukin-1 and Toll-like receptors. We identify here that Tollip interacts with Smad7, a major modulatory protein involved in the negative regulation of TGF-β signaling. Overexpression of Tollip antagonizes TGF-β-stimulated transcriptional response, Smad2 phosphorylation, and epithelial-mesenchymal transition. Tollip also interacts with ubiquitinated TβRI, and such interaction requires ubiquitin-associated domains of Tollip. The interaction and intracellular colocalization of Tollip with TβRI is enhanced by Smad7. Overexpression of Tollip accelerates protein degradation of activated TβRI. In addition, Tollip alters subcellular compartmentalization and endosomal trafficking of activated TβRI. Collectively, our studies reveal that Tollip cooperates with Smad7 to modulate intracellular trafficking and degradation of ubiquitinated TβRI, whereby negatively regulates TGF-β signaling pathway.

3.1902 SIRT5 deacetylates and activates urate oxidase in liver mitochondria of mice
Nakamura, Y., Ogura, M., Ogura, K., Tanaka, D. and Inagaki, N.
FEBS Lett., ***586***, *4076-4081 (2012)*

We identified urate oxidase (UOX) as a target of SIRT5 by comparing mitochondrial proteins in livers of SIRT5-overexpressing transgenic (SIRT5 Tg) and wild-type mice by using two-dimensional electrophoresis. Acetylation levels of UOX in liver of SIRT5 Tg mice were approximately half of those in wild-type mice, and UOX activity was significantly increased. *Invitro*-synthesized UOX protein was acetylated when incubated with mitochondria from wild-type mice liver but the levels were less when incubated with those from SIRT5 Tg mice liver. These results suggest that SIRT5 activates UOX through deacetylation in mouse liver mitochondria.

3.1903 Potent Inhibition of Late Stages of Hepadnavirus Replication by a Modified Cell Penetrating Peptide
Abdul, F., Ndeboko, B., Buronfosse, T., Zoulim, F., Kann, M., Nielsen, P.E. and Cova, L.
PloS One, ***7(11)***, *e48721 (2012)*

Cationic cell-penetrating peptides (CPPs) and their lipid domain-conjugates (CatLip) are agents for the delivery of (uncharged) biologically active molecules into the cell. Using infection and transfection assays we surprisingly discovered that CatLip peptides were able to inhibit replication of Duck Hepatitis B Virus (DHBV), a reference model for human HBV. Amongst twelve CatLip peptides we identified Deca-$(Arg)_8$ having a particularly potent antiviral activity, leading to a drastic inhibition of viral particle secretion without detectable toxicity. Inhibition of virion secretion was correlated with a dose-dependent increase in intracellular viral DNA. Deca-$(Arg)_8$ peptide did neither interfere with DHBV entry, nor with formation of mature nucleocapsids nor with their travelling to the nucleus. Instead, Deca-$(Arg)_8$ caused envelope protein accumulation in large clusters as revealed by confocal laser scanning microscopy indicating severe structural changes of preS/S. Sucrose gradient analysis of supernatants from Deca-$(Arg)_8$-treated cells showed unaffected naked viral nucleocapsids release, which was concomitant with a complete arrest of virion and surface protein-containing subviral particle secretion. This is the first report showing that a CPP is able to drastically block hepadnaviral release from infected cells by altering late stages of viral morphogenesis *via* interference with enveloped particle formation, without affecting naked nucleocapsid egress, thus giving a view inside the mode of inhibition. Deca-$(Arg)_8$ may be a useful tool for elucidating the hepadnaviral secretory pathway, which is not yet fully understood. Moreover we provide the first evidence that a modified CPP displays a novel antiviral mechanism targeting another step of viral life cycle compared to what has been so far described for other enveloped viruses.

3.1904 Three-Dimensional Architecture of the Rod Sensory Cilium and Its Disruption in Retinal Neurodegeneration

Gilliam, J.C., Chang, J.T., Sandoval, I.M., Zhang, Y., Li, T., Pittler, S.J., Chiu, W. and Wensel, T.G.
Cell, ***151(5)***, *1029-1041 (2012)*

Defects in primary cilia lead to devastating disease because of their roles in sensation and developmental signaling but much is unknown about ciliary structure and mechanisms of their formation and maintenance. We used cryo-electron tomography to obtain 3D maps of the connecting cilium and adjacent cellular structures of a modified primary cilium, the rod outer segment, from wild-type and genetically defective mice. The results reveal the molecular architecture of the cilium and provide insights into protein functions. They suggest that the ciliary rootlet is involved in cellular transport and stabilizes the axoneme. A defect in the BBSome membrane coat caused defects in vesicle targeting near the base of the cilium. Loss of the proteins encoded by the *Cngb1* gene disrupted links between the disk and plasma membranes. The structures of the outer segment membranes support a model for disk morphogenesis in which basal disks are enveloped by the plasma membrane.

3.1905 Complete failure of insulin-transmitted signaling, but not obesity-induced insulin resistance, impairs respiratory chain function in muscle

Franko, A., von Kleist-Retzow, J.C., Böse, M., Sanchez-Lasheras, C., Brodesser, S., Krut, O., Kunz, W.S., Wiedermann, D., Hoehn, M., Stöhr, O., Moll, L., Freude, S., Krone, W., Schubert, M. and Wiesner, R.J.
J. Mol. Med., ***90***, *1145-1160 (2012)*

The role of mitochondrial dysfunction in the development of insulin resistance and type 2 diabetes remains controversial. In order to specifically define the relationship between insulin receptor (InsR) signaling, insulin resistance, hyperglycemia, hyperlipidemia and mitochondrial function, we analyzed mitochondrial performance of insulin-sensitive, slow-oxidative muscle in four different mouse models. In obese but normoglycemic ob/ob mice as well as in obese but diabetic mice under high-fat diet, mitochondrial performance remained unchanged even though intramyocellular diacylglycerols (DAGs), triacylglycerols (TAGs), and ceramides accumulated. In contrast, in muscle-specific InsR knockout (MIRKO) and streptozotocin (STZ)-treated hypoinsulinemic, hyperglycemic mice, levels of mitochondrial respiratory chain complexes and mitochondrial function were markedly reduced. In STZ, but not in MIRKO mice, this was caused by reduced transcription of mitochondrial genes mediated via decreased PGC-1α expression. We conclude that mitochondrial dysfunction is not causally involved in the pathogenesis of obesity-associated insulin resistance under normoglycemic conditions. However, obesity-associated type 2 diabetes and accumulation of DAGs or TAGs is not associated with impaired mitochondrial function. In contrast, chronic hypoinsulinemia and hyperglycemia as seen in STZ-treated mice as well as InsR deficiency in muscle of MIRKO mice lead to mitochondrial dysfunction. We postulate that decreased mitochondrial mass and/or performance in skeletal muscle of non-diabetic, obese or type 2 diabetic, obese patients observed in clinical studies must be explained by genetic predisposition, physical inactivity, or other still unknown factors.

3.1906 Lysosomal delivery of therapeutic enzymes in cell models of Fabry disease

Marchesan, D., Cox, T.M. and Deegan, P.B.
J. Inherit. Metab. Dis., ***35(6)***, *1107-1117 (2012)*

The success of enzymatic replacement in Gaucher disease has stimulated development of targeted protein replacement for other lysosomal disorders, including Anderson-Fabry disease, which causes fatal cardiac, cerebrovascular and renal injury: deficiency of lysosomal α-Galactosidase A induces accumulation of glycosphingolipids. Endothelial cell storage was the primary endpoint in a clinical trial that led to market authorization. Two α-Galactosidase A preparations are licensed worldwide, but fatal outcomes persist, with storage remaining in many tissues. We compare mechanisms of uptake of α -Galactosidase A into cells relevant to Fabry disease, in order to investigate if the enzyme is targeted to the lysosomes in a mannose-6-phosphate receptor dependent fashion, as generally believed. α -Galactosidase A uptake was examined in fibroblasts, four different endothelial cell models, and hepatic cells in vitro. Uptake of europium-labeled human α -Galactosidase A was measured by time-resolved fluorescence. Ligand-specific uptake was quantified in inhibitor studies. Targeting to the lysosome was determined by precipitation and by confocal microscopy. The quantity and location of cation-independent mannose-6-phosphate receptors in the different cell models were investigated using confocal microscopy. Uptake and delivery of α -

Galactosidase A to lysosomes in fibroblasts is mediated by the canonical mannose-6-phosphate receptor pathway, but in endothelial cells in vitro this mechanism does not operate. Moreover, this observation is supported by a striking paucity of expression of cation independent mannose-6-phosphate receptors on the plasma membrane of the four endothelial cell models and by little delivery of enzyme to lysosomes, when compared with fibroblasts. If these observations are confirmed in vivo, alternative mechanisms will be needed to explain the ready clearance of storage from endothelial cells in patients undergoing enzyme replacement therapy.

3.1907 Mitochondrial Localization of ABC Transporter ABCG2 and Its Function in 5-Aminolevulinic Acid-Mediated Protoporphyrin IX Accumulation

Kobuchi, H., Moriya, K., Ogino, T., Fujita, H., Inoue, K., Shuin, T., Yasuda, T., Utsumi, K. and utsumi, T.
PloS One, ***7(11)***, *e50082 (2012)*

Accumulation of protoporphyrin IX (PpIX) in malignant cells is the basis of 5-aminolevulinic acid (ALA)-mediated photodynamic therapy. We studied the expression of proteins that possibly affect ALA-mediated PpIX accumulation, namely oligopeptide transporter-1 and -2, ferrochelatase and ATP-binding cassette transporter G2 (ABCG2), in several tumor cell lines. Among these proteins, only ABCG2 correlated negatively with ALA-mediated PpIX accumulation. Both a subcellular fractionation study and confocal laser microscopic analysis revealed that ABCG2 was distributed not only in the plasma membrane but also intracellular organelles, including mitochondria. In addition, mitochondrial ABCG2 regulated the content of ALA-mediated PpIX in mitochondria, and Ko143, a specific inhibitor of ABCG2, enhanced mitochondrial PpIX accumulation. To clarify the possible roles of mitochondrial ABCG2, we characterized stably transfected-HEK (ST-HEK) cells overexpressing ABCG2. In these ST-HEK cells, functionally active ABCG2 was detected in mitochondria, and treatment with Ko143 increased ALA-mediated mitochondrial PpIX accumulation. Moreover, the mitochondria isolated from ST-HEK cells exported doxorubicin probably through ABCG2, because the export of doxorubicin was inhibited by Ko143. The susceptibility of ABCG2 distributed in mitochondria to proteinase K, endoglycosidase H and peptide-*N*-glycosidase F suggested that ABCG2 in mitochondrial fraction is modified by *N*-glycans and trafficked through the endoplasmic reticulum and Golgi apparatus and finally localizes within the mitochondria. Thus, it was found that ABCG2 distributed in mitochondria is a functional transporter and that the mitochondrial ABCG2 regulates ALA-mediated PpIX level through PpIX export from mitochondria to the cytosol.

3.1908 In-depth analysis of the secretome identifies three major independent secretory pathways in differentiating human myoblasts

Le Bihan, M-C., Bigot, A., Jensen, S.S., Dennis, J.L., Rogowska-Wrzesinska, A., Laine, J., GAche, V., Furling, D., Nørregaard-jensen, O., Voit, T., Mouly, V., Coulton, G.R. and Butler-Browne, G.
J. Proteomics., ***77***, *344-356 (2012)*

Efficient muscle regeneration requires cross talk between multiple cell types via secreted signaling molecules. However, as yet there has been no comprehensive analysis of this secreted signaling network in order to understand how it regulates myogenesis in humans.
Using integrated proteomic and genomic strategies, we show that human muscle cells release not only soluble secreted proteins through conventional secretory mechanisms but also complex protein and nucleic acid cargos via membrane microvesicle shedding. The soluble secretome of muscle cells contains 253 conventionally secreted signaling proteins, including 43 previously implicated in myogenesis, while others are known to modulate various cell types thus implying a much broader role for myoblasts in muscle remodeling. We also isolated and characterized two types of secreted membrane-derived vesicles: nanovesicles harboring typical exosomal features and larger, morphologically distinct, microvesicles. While they share some common features, their distinct protein and RNA cargos suggest independent functions in myogenesis. We further demonstrate that both types of microvesicles can dock and fuse with adjacent muscle cells but also deliver functional protein cargo.
Thus, the intercellular signaling networks invoked during muscle differentiation and regeneration may employ conventional soluble signaling molecules acting in concert with muscle derived microvesicles delivering their cargos directly into target cells.

3.1909 The Ubiquitin Ligase Synoviolin Up-regulates Amyloid β Production by Targeting a Negative Regulator of -Secretase, Rer1, for Degradation

Tanabe, C., Maeda, T., Zou, K., Liu, J., Nakajima, T. and Komano, H.
J. Biol. Chem., ***287(53)***, *44203-44211 (2012)*

Alzheimer's disease is characterized by the deposition of Aβ, which is generated from the amyloid precursor protein through its cleavage by β- and γ-secretases. The γ-secretase complex component nicastrin (NCT) plays significant roles in the assembly and proper trafficking of the γ-secretase complex and in the recognition of amyloid precursor protein. NCT is incorporated into the γ-secretase complex in the endoplasmic reticulum (ER) and glycosylated in the Golgi. In contrast, unassembled NCT is retrieved or retained in the ER by the protein Retention in endoplasmic reticulum 1 (Rer1). We reported previously that synoviolin (Syvn), an E3 ubiquitin ligase, degrades NCT and affects the generation of Aβ. Here, we examined in more detail the effect of Syvn on the generation of Aβ. We found that overexpression of a dominant negative form of Syvn (C307A mutant) and a Syvn-RNAi decreased the generation of Aβ. These results indicate that the ubiquitin ligase activity of Syvn up-regulates the generation of Aβ. We hypothesized, therefore, that Syvn regulates the assembly or localization of the γ-secretase complex by ubiquitinating Rer1, resulting in its subsequent degradation. Our findings that the level of Rer1 was increased in Syvn knockout fibroblasts because of inhibition of its degradation support this hypothesis. Moreover, we found that Rer1 interacts with Syvn in the ER, is ubiquitinated by Syvn, and is then degraded via the proteasome or lysosomal pathways. Finally, we showed that localization of mature NCT to the plasma membrane as well as γ-secretase complex levels are decreased in fibroblasts of Syvn knockout mice. Thus, it is likely that Syvn regulates the assembly of the γ-secretase complex via the degradation of Rer1, which results in the generation of Aβ.

3.1910 Sphingosine 1-Phosphate (S1P) Carrier-dependent Regulation of Endothelial Barrier: HIGH DENSITY LIPOPROTEIN (HDL)-S1P PROLONGS ENDOTHELIAL BARRIER ENHANCEMENT AS COMPARED WITH ALBUMIN-S1P VIA EFFECTS ON LEVELS, TRAFFICKING, AND SIGNALING OF S1P1

Wilkerson, B.A., Grass, G.D., Wing, S.B., Argraves, W.S. and Argraves, K.M.
J. Biol. Chem., ***287(53)***, *44645-44653 (2012)*

Sphingosine 1-phosphate (S1P) is a blood-borne lysosphingolipid that acts to promote endothelial cell (EC) barrier function. In plasma, S1P is associated with both high density lipoproteins (HDL) and albumin, but it is not known whether the carriers impart different effects on S1P signaling. Here we establish that HDL-S1P sustains EC barrier longer than albumin-S1P. We showed that the sustained barrier effects of HDL-S1P are dependent on signaling by the S1P receptor, S1P1, and involve persistent activation of Akt and endothelial NOS (eNOS), as well as activity of the downstream NO target, soluble guanylate cyclase (sGC). Total S1P1 protein levels were found to be higher in response to HDL-S1P treatment as compared with albumin-S1P, and this effect was not associated with increased S1P1 mRNA or dependent on *de novo* protein synthesis. Several pieces of evidence indicate that long term EC barrier enhancement activity of HDL-S1P is due to specific effects on S1P1 trafficking. First, the rate of S1P1 degradation, which is proteasome-mediated, was slower in HDL-S1P-treated cells as compared with cells treated with albumin-S1P. Second, the long term barrier-promoting effects of HDL-S1P were abrogated by treatment with the recycling blocker, monensin. Finally, cell surface levels of S1P1 and levels of S1P1 in caveolin-enriched microdomains were higher after treatment with HDL-S1P as compared with albumin-S1P. Together, the findings reveal S1P carrier-specific effects on S1P1 and point to HDL as the physiological mediator of sustained S1P1-PI3K-Akt-eNOS-sGC-dependent EC barrier function.

3.1911 Vigilin interacts with signal peptide peptidase

Lu, S.H-J., Jeon, A.H.W., Schmitt-Ulms, G., Qamar, S., Dodd, R., McDonald, B., Li, Y., Meadows, W., Cox, K., Bohm, C., Chen, F., fraser, P. and St. George-Hyslop, P.
Proteome Science, ***10(33)*** *(2012)*

Background
Signal peptide peptidase (SPP), a member of the presenilin-like intra-membrane cleaving aspartyl protease family, migrates on Blue Native (BN) gels as 100 kDa, 200 kDa and 450 kDa species. SPP has recently been implicated in other non-proteolytic functions such as retro-translocation of MHC Class I molecules and binding of misfolded proteins in the endoplasmic reticulum (ER). These high molecular weight SPP complexes might contain additional proteins that regulate the proteolytic activity of SPP or support its non-catalytic functions.
Results
In this study, an unbiased iTRAQ-labeling mass spectrometry approach was used to identify SPP-interacting proteins. We found that vigilin, a ubiquitous multi-KH domain containing cytoplasmic protein involved in RNA binding and protein translation control, selectively enriched with SPP. Vigilin interacted

with SPP and both proteins co-localized in restricted intracellular domains near the ER, biochemically co-fractionated and were part of the same 450 kDa complex on BN gels. However, vigilin does not alter the protease activity of SPP, suggesting that the SPP-vigilin interaction might be involved in the non-proteolytic functions of SPP.

Conclusions

We have identified and validated vigilin as a novel interacting partner of SPP that could play an important role in the non-proteolytic functions of SPP. This data adds further weight to the idea that intramembrane-cleaving aspartyl proteases, such as presenilin and SPPs, could have other functions besides the proteolysis of short membrane stubs.

3.1912 Ligand-independent activation of EphA2 by arachidonic acid induces metastasis-like behaviour in prostate cancer cells

Tawadros, T., Brown, M.D., Hart, C.A. and Clarke, N.W.
*Br. J. Cancer, **107(10)**, 1737-1744 (2012)*

Background:
High intake of omega-6 polyunsaturated fatty acids (PUFA) has been associated with clinical progression in prostate cancer (CaP). This study investigates the signalling mechanism by which the omega-6 PUFA arachidonic acid (AA) induces prostatic cellular migration to bone marrow stroma.
Methods:
Western blot analysis of the PC-3, PC3-GFP, DU 145 and LNCaP cells or their lipid raft (LR) components post AA stimulation was conducted in association with assays for adhesion and invasion through the bone marrow endothelial monolayers.
Results:
Arachidonic acid increased transendothelial migration of PC3-GFP cells (adhesion 37%±0.08, *P*=0.0124; transmigration 270%±0.145, *P*=0.0008). Akt, Src and focal adhesion kinase (FAK) pathways were induced by AA and integrally involved in transendothelial migration. LR were critical in AA uptake and induced Akt activity. Ephrin receptor A2 (EphA2), localised in LR, is expressed in DU 145 and PC-3 cells. Arachidonic acid induced a rapid increase of EphA2 Akt-dependent/ligand-independent activation, while knockdown of the EphrinA1 ligand decreased AA induced transendothelial migration, with an associated decrease in Src and FAK activity. Arachidonic acid activated Akt in EphA2$^-$ LNCaP cells but failed to induce BMEC transendothelial invasion.
Conclusion:
Arachidonic acid induced stimulation of EphA2 *in vitro* is associated fundamentally with CaP epithelial migration across the endothelial barrier.

3.1913 Exosome-mediated delivery of siRNA in vitro and in vivo

El-Andaloussi, S., Lee, Y., Lakhal-Littleton, S., Li, J., Seow, Y., Gardiner, C., Alvarez-Erviti, L., Sargent, I.L. and Wood, M.J.A.
*Nature Protocols, **7(12)**, 2112-2126 (2012)*

The use of small interfering RNAs (siRNAs) to induce gene silencing has opened a new avenue in drug discovery. However, their therapeutic potential is hampered by inadequate tissue-specific delivery. Exosomes are promising tools for drug delivery across different biological barriers. Here we show how exosomes derived from cultured cells can be harnessed for delivery of siRNA *in vitro* and *in vivo*. This protocol first describes the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. Next, we explain how to purify and characterize exosomes from transfected cell supernatant. Next, we detail crucial steps for loading siRNA into exosomes. Finally, we outline how to use exosomes to efficiently deliver siRNA *in vitro* and *in vivo* in mouse brain. Examples of anticipated results in which exosome-mediated siRNA delivery is evaluated by functional assays and imaging are also provided. The entire protocol takes ~3 weeks.

3.1914 Down-regulation of alpha-2u globulin in renal mitochondria of STZ-induced diabetic rats observed by a proteomic method

Sun, S-H., Liu, S-Q., Cai, C-P., Cai, R., Chen, L. and Zhang, Q-B.
*Annales d'Endocrinologie, **73(6)**, 530-541 (2012)*

Aim
To identify the changes of mitochondrial protein expression in diabetic renal parenchyma and to characterize their molecular functions and biological processes in diabetes.

Methods
Mitochondrial proteins extracted from renal parenchyma mitochondria of streptozotocin-induced diabetic rats and normal rats were separated by two-dimensional polyacrylamide gel electrophoresis and identified by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry.
Results
Eleven proteins from 533 visualized protein spots displayed significant different expressions in mitochondria of diabetic kidneys compared with those in normal ones. Among these altered proteins, two proteins with the most obvious changes in protein expression were identified as alpha-2u globulin (mature protein, named A2) and its proteolytically modified form (named A2-fragment) respectively. These proteins were found in mitochondria of male rat renal parenchyma and were proved to be down-regulated in diabetic rats simultaneously.
Conclusion
Our results suggest that down-regulation of alpha-2u globulin may be associated with an abnormal β-oxidation of long-chain fatty acids during diabetes. The decreased expression of A2-fragment in renal mitochondria of diabetic nephropathy may reduce fatty acid β-oxidation, which leads to a diminished energy supply from mitochondria to kidney tissue and the deposition of a large number of fatty acids in the kidney, ultimately causing and aggravating kidney damage. In conclusion, these findings may be helpful for understanding the molecular mechanism of diabetic nephropathy.

3.1915 Dual Effects of Statins on Aβ Metabolism: Upregulation of the Degradation of APP-CTF and Aβ Clearance
Sato, N., Shinohara, M., Rakugi, H. and Morishita, R.
Neurodegenerative Dis., ***10(1-4)***, *305-308 (2012)*

Background/Aims: Retrospective cohort studies have suggested that statin users have a lower prevalence of dementia. On the other hand, a randomized controlled study failed to show beneficial effects on the cognitive decline in Alzheimer's disease (AD). However, a prospective cohort study demonstrated that users of statins had a lower incidence of AD. One possible interpretation might be that statins could prevent or delay the onset of AD, but not slow cognitive decline once the disease has set in. Given that statins could prevent or delay the onset of AD, what is the responsible mechanism? *Methods:* We investigated the effect of fluvastatin on Aβ metabolism at a clinically relevant dose in mice. *Results:* Fluvastatin reduced the brain Aβ level by increased trafficking of the carboxyl terminal fragment of the amyloid precursor protein (APP-CTF), which was mediated by inhibition of protein isoprenylation. Moreover, the statin reduced the brain Aβ level through enhanced Aβ clearance mediated by upregulation of low-density lipoprotein receptor-related protein 1 (LRP-1) expression. The statin increased LRP-1 expression, mediated by inhibition of protein isoprenylation. *Conclusion:* Statins might prevent the onset of AD through reduced Aβ production by enhancement of APP-CTF degradation and/or upregulation of Aβ clearance. We also showed that promotion of APP-CTF degradation and upregulation of Aβ clearance could be modified by a drug, suggesting possible mechanistic targets for disease-modifying drugs.

3.1916 Autophagosomes induced by a bacterial Beclin 1 binding protein facilitate obligatory intracellular infection
Niu, H., Xiong, Q., Yamamoto, A., Hayashi-Nishino, M. and Rikihisa, Y.
PNAS, ***109(51)***, *20800-20807 (2012)*

Autophagy, a cytoplasmic catabolic process, plays a critical role in defense against intracellular infection. In turn, evasion or inhibition of autophagy has emerged as an important virulence factor for intracellular pathogens. However, *Anaplasma phagocytophilum*, the obligatory intracellular bacterium that causes human granulocytic anaplasmosis, replicates in the membrane-bound compartment resembling early autophagosome. Here, we found that *Anaplasma* translocated substrate 1 (Ats-1), a type IV secretion effector, binds Beclin 1, a subunit of the class III PI3K and Atg14L, and it nucleates autophagosomes with markers of omegasomes, double FYVE-containing protein 1, Atg14L, and LC3. Ats-1 autophagy induction did not activate the starvation signaling pathway of mammalian target of rapamycin. These autophagy proteins were also localized to the *Anaplasma* inclusion. Ectopically expressed Ats-1 targeted the *Anaplasma* inclusions and enhanced infection, whereas host cytoplasmic delivery of anti–Ats-1 or Beclin 1 depletion by siRNA suppressed the infection; *beclin 1* heterozygous-deficient mice were resistant to *Anaplasma* infection. Furthermore, *Anaplasma* growth arrest by the class III PI3K inhibitor 3-methyladenine was alleviated by essential amino acid supplementation. Thus, *Anaplasma* actively induces autophagy by secreting Ats-1 that hijacks the Beclin 1-Atg14L autophagy initiation pathway likely to acquire host nutrients for its growth.

3.1917 **Proteome Analysis of Cry4Ba Toxin-interacting Aedes aegypti Lipid Rafts using geLC–MS/MS**
Bayyareddy, K., Zhu, X., Orlando, r. and Adang, M.J.
J. Proteome Res., ***11(12)***, *5843-5855 (2012)*

Lipid rafts are microdomains in the plasma membrane of eukaryotic cells. Among their many functions, lipid rafts are involved in cell toxicity caused by pore forming bacterial toxins including *Bacillus thuringiensis* (Bt) Cry toxins. We isolated lipid rafts from brush border membrane vesicles (BBMV) of *Aedes aegypti* larvae as a detergent resistant membrane (DRM) fraction on density gradients. Cholesterol, aminopeptidase (APN), alkaline phosphatase (ALP) and the raft marker flotillin were preferentially partitioned into the lipid raft fraction. When mosquitocidal Cry4Ba toxin was preincubated with BBMV, Cry4Ba localized to lipid rafts. A proteomic approach based on one-dimensional gel electrophoresis, in-gel trypsin digestion, followed by liquid chromatography–mass spectrometry (geLC–MS/MS) identified a total of 386 proteins. Of which many are typical lipid raft marker proteins including flotillins and glycosylphosphatidylinositol (GPI)-anchored proteins. Identified raft proteins were annotated *in silico* for functional and physicochemical characteristics. Parameters such as distribution of isoelectric point, molecular mass, and predicted post-translational modifications relevant to lipid raft proteins (GPI anchorage and myristoylation or palmitoylation) were analyzed for identified proteins in the DRM fraction. From a functional point of view, this study identified proteins implicated in Cry toxin interactions as well as membrane-associated proteins expressed in the mosquito midgut that have potential relevance to mosquito biology and vector management.

3.1918 **MeCP2 Binds to 5hmC Enriched within Active Genes and Accessible Chromatin in the Nervous System**
Mellen, M., Ayata, P., Dewell, S., Kriaucionis, S. and Heintz, N.
Cell, ***151(7)***, *1417-1430 (2012)*

The high level of 5-hydroxymethylcytosine (5hmC) present in neuronal genomes suggests that mechanisms interpreting 5hmC in the CNS may differ from those present in embryonic stem cells. Here, we present quantitative, genome-wide analysis of 5hmC, 5-methylcytosine (5mC), and gene expression in differentiated CNS cell types in vivo. We report that 5hmC is enriched in active genes and that, surprisingly, strong depletion of 5mC is observed over these regions. The contribution of these epigenetic marks to gene expression depends critically on cell type. We identify methyl-CpG-binding protein 2 (MeCP2) as the major 5hmC-binding protein in the brain and demonstrate that MeCP2 binds 5hmC- and 5mC-containing DNA with similar high affinities. The Rett-syndrome-causing mutation R133C preferentially inhibits 5hmC binding. These findings support a model in which 5hmC and MeCP2 constitute a cell-specific epigenetic mechanism for regulation of chromatin structure and gene expression.

3.1919 **Phosphorylation of claudin-2 on serine 208 promotes membrane retention and reduces trafficking to lysosomes**
Van Itallie, C.M., Tietgens, A.J., LoGrande, K., Aponte, A., Gucek, M. and Anderson, J.M.
J. Cell Sci., ***125(20)***, *4902-4912 (2012)*

Claudins are critical components of epithelial and endothelial tight junction seals, but their post-transcriptional regulation remains poorly understood. Several studies have implicated phosphorylation in control of claudin localisation and/or function, but these have focused on single sites or pathways with differing results, so that it has been difficult to draw general functional conclusions. In this study, we used mass spectrometry (MS) analysis of purified claudin-2 from MDCK II cells and found that the cytoplasmic tail is multiply phosphorylated on serines, a threonine and tyrosines. Phos-tag SDS PAGE revealed that one site, S208, is heavily constitutively phosphorylated in MDCK II cells and in mouse kidney; this site was targeted for further study. Mutational analysis revealed that the phosphomimetic mutant of claudin-2, S208E, was preferentially localised to the plasma membrane while claudin-2 S208A, which could not be phosphorylated at this site, both immunolocalized and co-fractionated with lysosomal markers. Mutations at sites that were previously reported to interfere with plasma membrane targeting of claudin-2 reduced phosphorylation at S208, suggesting that membrane localisation is required for phosphorylation; however phosphorylation at S208 did not affect binding to ZO-1 or ZO-2 Administration of forskolin or PGE2 resulted in dephosphorylation at S208 and transient small increases in transepithelial electrical resistance (TER). Together these data are consistent with phosphorylation at S208 playing a major role in the retention of claudin-2 at the plasma membrane.

3.1920 The nuclear translocation of endostatin is mediated by its receptor nucleolin in endothelial cells
Song, N., Ding, Y., Zhuo, W., He, T., Fu, Z., Chen, Y., Song, X., Fu, Y. and Luo, Y.
Angiogenesis, ***15***, *697-711 (2012)*

Endostatin, the C-terminal fragment of collagen XVIII, is a potent anti-angiogenic factor that significantly modulates the gene expression pattern in endothelial cells. Upon cell surface binding, endostatin can not only function extracellularly, but also translocate to the nucleus within minutes. However, the mechanism by which this occurs is partially understood. Here we systematically investigated the nuclear translocation mechanism of endostatin. By chemical inhibition and RNA interference, we firstly observed that clathrin-mediated endocytosis, but not caveolae-dependent endocytosis or macropinocytosis, is essential for the nuclear translocation of endostatin. We then indentified that nucleolin and integrin α5β1, two widely accepted endostatin receptors, mediate this clathrin-dependent uptake process, which also involves urokinase plasminogen activator receptor (uPAR). Either mutagenesis study, fluorescence resonance energy transfer assay, or fluorescence cell imaging demonstrates that nucleolin and integrin α5β1 interact with uPAR simultaneously upon endostatin stimulation. Blockade of uPAR decreases not only the interaction between nucleolin and integrin α5β1, but also the uptake process, suggesting that the nucleolin/uPAR/integrin α5β1 complex facilitates the internalization of endostatin. After endocytosis, nucleolin further regulates the nuclear transport of endostatin. RNA interference and mutational analysis revealed that the nuclear translocation of endostatin involves the association of nucleolin with importin α1β1 via the nuclear localization sequence. Taken together, this study reveals the pathway by which endostatin translocates to the nucleus and the importance of nucleolin in this process, providing a new perspective for the functional investigation of the nuclear-translocated endostatin in endothelial cells.

3.1921 Intracellular Delivery and Trafficking Dynamics of a Lymphoma-Targeting Antibody–Polymer Conjugate
Berguig, G.Y., Convertine, A.J., Shi, J., Palanca-Wessels, M.C., Duvall, C.L., Pun, S.H., Press, O.W. and Stayton, P.S.
Mol. Pharmaceutics, ***9(12)***, *3506-3514 (2012)*

Ratiometric fluorescence and cellular fractionation studies were employed to characterize the intracellular trafficking dynamics of antibody–poly(propylacrylic acid) (PPAA) conjugates in CD22+ RAMOS-AW cells. The HD39 monoclonal antibody (mAb) directs CD22-dependent, receptor-mediated uptake in human B-cell lymphoma cells, where it is rapidly trafficked to the lysosomal compartment. To characterize the intracellular-release dynamics of the polymer–mAb conjugates, HD39-streptavidin (HD39/SA) was dual-labeled with pH-insensitive Alexa Fluor 488 and pH-sensitive pHrodo fluorophores. The subcellular pH distribution of the HD39/SA–polymer conjugates was quantified as a function of time by live-cell fluorescence microscopy, and the average intracellular pH value experienced by the conjugates was also characterized as a function of time by flow cytometry. PPAA was shown to alter the intracellular trafficking kinetics strongly relative to HD39/SA alone or HD39/SA conjugates with a control polymer, poly(methacryclic acid) (PMAA). Subcellular trafficking studies revealed that after 6 h, only 11% of the HD39/SA–PPAA conjugates had been trafficked to acidic lysosomal compartments with values at or below pH 5.6. In contrast, the average intracellular pH of HD39/SA alone dropped from 6.7 ± 0.2 at 1 h to 5.6 ± 0.5 after 3 h and 4.7 ± 0.6 after 6 h. Conjugation of the control polymer PMAA to HD39/SA showed an average pH drop similar to that of HD39/SA. Subcellular fractionation studies with tritium-labeled HD39/SA demonstrated that after 6 h, 89% of HD39/SA was associated with endosomes (Rab5+) and lysosomes (Lamp2+), while 45% of HD39/SA–PPAA was translocated to the cytosol (lactate dehydrogenase+). These results demonstrate the endosomal-releasing properties of PPAA with antibody–polymer conjugates and detail their intracellular trafficking dynamics and subcellular compartmental distributions over time.

3.1922 Nitric oxide induces segregation of decay accelerating factor (DAF or CD55) from the membrane lipid-rafts and its internalization in human endometrial cells
Banadakoppa, M., Goluszko, P., Liebenthal, D. and Yallampalli, C.
Cell Biol. Int., ***36(10)***, *901-907 (2012)*

Recent studies suggest that DAF (decay accelerating factor), a complement regulatory protein, present in lipid rafts, is utilized by Dr fimbriated *Escherichia coli* for their binding and internalization. Previous studies in our laboratory have shown that NO (nitric oxide) can reduce the invasion of Dr(+) *E. coli* and the severity of uterine infection in pregnant rats. Also, the expression level of DAF both at the mRNA and protein levels has been shown to be reduced by NO. Therefore NO mediated down-regulation of DAF

appears to be an important factor in reducing the susceptibility to *E. coli* infection. However, it is unclear if NO can actually modulate the membrane association of DAF and therefore initial bacterial binding to cells. We found that NO induces the delocalization of DAF from the G_{M1}-rich lipid rafts. Using biochemical and cell biological approaches in a uterine epithelial cell model (Ishikawa cells), DAF accumulates in caveolae upon exposure to NO. Interaction of DAF with the caveolar protein, caveolin1, leads to their internalization by endosomes. NO-induced delocalization of DAF from the lipid raft and its accumulation in caveolae are mediated through a cGMP (cyclic guanosine monophosphate) pathway. The acute localized synthesis of NO and its influence on DAF localization may represent an important unrecognized phenomenon of host defence against Dr(+) *E. coli* bacteria, as well as many disease conditions that involve complement system.

3.1923 Cis-9,trans-11-conjugated linoleic acid affects lipid raft composition and sensitizes human colorectal adenocarcinoma HT-29 cells to X-radiation

Gradzka, I., Sochanowicz, B., Brzoska, K., Wojciuk, G., Sommer, S., Wojewodzka, M., Gasinska, A., Degen, C., Jahreis, G. and Szumiel, I.
Biochim. Biophys. Acta, ***1830****, 2233-2242 (2013)*

Background
Investigations concerned the mechanism of HT-29 cells radiosensitization by cis-9,trans-11-conjugated linoleic acid (c9,t11-CLA), a natural component of human diet with proven antitumor activity.
Methods
The cells were incubated for 24 h with 70 μ M c9,t11-CLA and then X-irradiated. The following methods were used: gas chromatography (incorporation of the CLA isomer), flow cytometry (cell cycle), cloning (survival), Western blotting (protein distribution in membrane fractions), and pulse-field gel electrophoresis (rejoining of DNA double-strand breaks). In parallel, DNA-PK activity, γ -H2AX foci numbers and chromatid fragmentation were estimated. Gene expression was analysed by RT-PCR and chromosomal aberrations by the mFISH method. Nuclear accumulation of the EGF receptor (EGFR) was monitored by ELISA.
Results and conclusions
C9,t11-CLA sensitized HT-29 cells to X-radiation. This effect was not due to changes in cell cycle progression or DNA-repair-related gene expression. Post-irradiation DSB rejoining was delayed, corresponding with the insufficient DNA-PK activation, although chromosomal aberration frequencies did not increase. Distributions of cholesterol and caveolin-1 in cellular membrane fractions changed. The nuclear EGFR translocation, necessary to increase the DNA-PK activity in response to oxidative stress, was blocked. We suppose that c9,t11-CLA modified the membrane structure, thus disturbing the intracellular EGFR transport and the EGFR-dependent pro-survival signalling, both functionally associated with lipid raft properties.
General Significance
The results point to the importance of the cell membrane interactions with the nucleus after injury inflicted by X -rays. Compounds like c9,t11-CLA, that specifically alter membrane properties, could be used to develop new anticancer strategies.

3.1924 Cell-surface glycosaminoglycans inhibit intranuclear uptake but promote post-nuclear processes of polyamidoamine dendrimer–pDNA transfection

Ziraksaz, Z., Normani, A., Ruponen, M., Soleimani, M., Tabbakhian, M. and Haririan, I.
Eur. J. Pharmaceut. Sci., ***48****, 55-63 (2013)*

Background
Interaction of cell-surface glycosaminoglycans (GAGs) with non-viral vectors seems to be an important factor which modifies the intracellular destination of the gene complexes. Intracellular kinetics of polyamidoamine (PAMAM) dendrimer as a non-viral vector in cellular uptake, intranuclear delivery and transgene expression of plasmid DNA with regard to the cell-surface GAGs has not been investigated until now.
Methods
The physicochemical properties of the PAMAM–pDNA complexes were characterized by photon correlation spectroscopy, atomic force microscopy, zeta measurement and agarose gel electrophoresis. The transfection efficiency and toxicity of the complexes at different nitrogen to phosphate (N:P) ratios were determined using various in vitro cell models such as human embryonic kidney cells, chinese hamster ovary cells and its mutants lacking cell-surface GAGs or heparan sulphate proteoglycans (HSPGs).

Cellular uptake, nuclear uptake and transfection efficiency of the complexes were determined using flow cytometry and optimized cell-nuclei isolation with quantitative real-time PCR and luciferase assay.
Results
Physicochemical studies showed that PAMAM dendrimer binds pDNA efficiently, forms small complexes with high positive zeta potential and transfects cells properly at N:P ratios around 5 and higher. The cytotoxicity could be a problem at N:Ps higher than 10. GAGs elimination caused nearly one order of magnitude higher pDNA nuclear uptake and more than 2.6-fold higher transfection efficiency than CHO parent cells. However, neither AUC of nuclear uptake, nor AUC of transfection affected significantly by only cell-surface HSPGs elimination and interesting data related to the effect of GAGs on intranuclear pDNA using PAMAM as delivery vector have been reported in this study.
Conclusion
Presented data shows that the rate-limiting step of PAMAM–pDNA complexes transfection is located after delivery to the cell nucleus and GAGs are regarded as an inhibitor of the intranuclear delivery step, while slightly promotes transgene expression.

3.1925 The function of the ATP-binding cassette (ABC) transporter ABCB1 is not susceptible to actin disruption
Meszaros, P., Hummel, I., Klappe, K., Draghiciu, O., Hoekstra, D. and Kok, J.W.
*Biochim. Biophys. Acta, **1828**, 340-351 (2013)*

Previously we have shown that the activity of the multidrug transporter ABCC1 (multidrug resistance protein 1), and its localization in lipid rafts, depends on cortical actin (Hummel I, Klappe K, Ercan C, Kok JW. Mol. Pharm. 2011 79, 229–40). Here we show that the efflux activity of the ATP-binding cassette (ABC) family member ABCB1 (P-glycoprotein), did not depend on actin, neither in ABCB1 over expressing murine National Institutes of Health (NIH) 3T3 MDR1 G185 cells nor in human SK-N-FI cells, which endogenously express ABCB1. Disruption of the actin cytoskeleton, upon treatment of the cells with latrunculin B or cytochalasin D, caused severe changes in cell and membrane morphology, and concomitant changes in the subcellular distribution of ABCB1, as revealed by confocal laser scanning and electron microscopy. Nevertheless, irrespective of actin perturbation, the cell surface pool of ABCB1 remained unaltered. In NIH 3T3 MDR1 G185 cells, ABCB1 is partly localized in detergent-free lipid rafts, which partitioned in two different density gradient regions, both enriched in cholesterol and sphingolipids. Interestingly, disruption of the actin cytoskeleton did not change the density gradient distribution of ABCB1. Our data demonstrate that the functioning of ABCB1 as an efflux pump does not depend on actin, which is due to its distribution in both cell surface-localized non-raft membrane areas and lipid raft domains, which do not depend on actin stabilization.

3.1926 A silicon cell cycle in a bacterial model of calcium phosphate mineralogenesis
Linton, K.M., Tapping, C.R., Adams, D.G., CarterR, D.H., Shore, R.C. and aaron, J.E:
*Micron, **44**, 419-432 (2013)*

The prokaryote *Corynebacterium matruchotii* produces calcium phosphate (bone salt) and may serve as a convenient model for examining individual factors relevant to vertebrate calcification. A factor of current clinical uncertainty is silicon. To investigate its possible role in biomineralisation advanced optical (digital deconvolution and 3D fluorescent image rendering) and electron microscopy (EDX microanalysis and elemental mapping) were applied to calcifying microbial colonies grown in graded Si concentrations (0–60 mM). Cell viability was confirmed throughout by TO-PRO-3-iodide and SYTO-9 nucleic acid staining. It was observed that calcium accumulated in dense intracellular microspherical objects (*types i–iii*) as nanoparticles (5 nm, *type i*), nanospheres (30–50 nm, *type* ii) and filamentous clusters (0.1–0.5 μm, *type* iii), with a regular transitory Si content evident. With bacterial colony development (7–28 days) the P content increased from 5 to 60%, while Si was displaced from 60 to 5%, distinguishing the phenomenon from random contamination, and with a significant relationship ($p < 0.001$) found between calcified object number and Si supplementation (optimum 0.01 mM). The Si-containing, intracellular calcified objects (also positive for Mg and negative with Lysensor blue DND-167 for acidocalcisomes) were extruded naturally in bubble-like chains to complete the cycle by coating the cell surface with discrete mineral particles. These could be harvested by lysis, French press and density fractionation when Si was confirmed in a proportion. It was concluded that the unexplained orthopaedic activity of Si may derive from its special property to facilitate calcium phosphorylation in biological systems, thereby recapitulating an ancient and conserved bacterial cycle of calcification via silicification.

3.1927 Biogenesis of the Vaccinia Virus Membrane: Genetic and Ultrastructural Analysis of the Contributions of the A14 and A17 Proteins

Unger, B., Mercer, J., Boyle, K.A.a nd Traktman, P.

J. Virol., ***87(2)***, *1083-1097 (2013)*

Vaccinia virus membrane biogenesis requires the A14 and A17 proteins. We show here that both proteins can associate with membranes co- but not posttranslationally, and we perform a structure function analysis of A14 and A17 using inducible recombinants. In the absence of A14, electron-dense virosomes and distinct clusters of small vesicles accumulate; in the absence of A17, small vesicles form a corona around the virosomes. When the proteins are induced at 12 h postinfection (hpi), crescents appear at the periphery of the electron-dense virosomes, with the accumulated vesicles likely contributing to their formation. A variety of mutant alleles of A14 and A17 were tested for their ability to support virion assembly. For A14, biologically important motifs within the N-terminal or central loop region affected crescent maturation and the immature virion (IV)→mature virion (MV) transition. For A17, truncation or mutation of the N terminus of A17 engendered a phenotype consistent with the N terminus of A17 recruiting the D13 scaffold protein to nascent membranes. When N-terminal processing was abrogated, virions attempted to undergo the IV-to-MV transition without removing the D13 scaffold and were therefore noninfectious and structurally aberrant. Finally, we show that A17 is phosphorylated exclusively within the C-terminal tail and that this region is a direct substrate of the viral F10 kinase. *In vivo*, the biological competency of A17 was reduced by mutations that prevented its serine-threonine phosphorylation and restored by phosphomimetic substitutions. Precleavage of the C terminus or abrogation of its phosphorylation diminished the IV→MV maturation; a block to cleavage spared virion maturation but compromised the yield of infectious virus.

3.1928 Gangliosides Have a Functional Role during Rotavirus Cell Entry

Martinez, M.A., Lopez, S., Arias, C.F. and Isa, P.

J. Virol., ***87(2)***, *1115-1122 (2013)*

Cell entry of rotaviruses is a complex process, which involves sequential interactions with several cell surface molecules. Among the molecules implicated are gangliosides, glycosphingolipids with one or more sialic acid (SA) residues. The role of gangliosides in rotavirus cell entry was studied by silencing the expression of two key enzymes involved in their biosynthesis—the UDP-glucose:ceramide glucosyltransferase (UGCG), which transfers a glucose molecule to ceramide to produce glucosylceramide GlcCer, and the lactosyl ceramide-α-2,3–sialyl transferase 5 (GM3-s), which adds the first SA to lactoceramide-producing ganglioside GM3. Silencing the expression of both enzymes resulted in decreased ganglioside levels (as judged by GM1a detection). Four rotavirus strains tested (human Wa, simian RRV, porcine TFR-41, and bovine UK) showed a decreased infectivity in cells with impaired ganglioside synthesis; however, their replication after bypassing the entry step was not affected, confirming the importance of gangliosides for cell entry of the viruses. Interestingly, viral binding to the cell surface was not affected in cells with inhibited ganglioside synthesis, but the infectivity of all strains tested was inhibited by preincubation of gangliosides with virus prior to infection. These data suggest that rotaviruses can attach to cell surface in the absence of gangliosides but require them for productive cell entry, confirming their functional role during rotavirus cell entry.

3.1929 Identification of lysosomal sialidase NEU1 and plasma membrane sialidase NEU3 in human erythrocytes

D'Avila, F., Tringali, C., Papini, N., Anastasia, L., Croci, g., Massaccesi, L., Monti, E., Tettamanti, G. and Venerando, B.

J. Cell. Biochem., ***114(1)***, *204-211 (2013)*

The sialylation level of molecules, sialoglycoproteins and gangliosides, protruding from plasma membranes regulates multiple facets of erythrocyte function, from interaction with endothelium to cell lifespan. Our results demonstrate that: (a) Both sialidases NEU1 and NEU3 are present on erythrocyte plasma membrane; (b) NEU1 is kept on the plasma membrane in absence of the protective protein/cathepsin A (PPCA); (c) NEU1 and NEU3 are retained on the plasma membrane, as peripheral proteins, associated to the external leaflet and released by alkaline treatments; (d) NEU1 and NEU3 are segregated in Triton X-100 detergent-resistant membrane domains (DRMs); (e) NEU3 shows activity also at neutral pH; and (f) NEU1 and NEU3 are progressively lost during erythrocyte life. Interestingly, sialidase activity released from erythrocyte membranes after an alkaline treatment preserves its functionality and recognizes sialoglycoproteins and gangliosides. On the other hand, the weak anchorage

of sialidases to the plasma membrane and their loss during erythrocyte life could be a tool to preserve the cellular sialic acid content in order to avoid the early ageing of erythrocyte and processes of cell aggregation in the capillaries.

3.1930 Sortilin and SorLA Display Distinct Roles in Processing and Trafficking of Amyloid Precursor Protein

Gustavsen, C., Glerup, S., Pallesen, L.T., Olsen, D., Andersen, O.M., Nykjær, A., Madsen, P. and Petersen, C.M.
J. Neurosci., ***33(1)***, *64-71 (2013)*

The development and progression of Alzheimer's disease is linked to excessive production of toxic amyloid-β peptide, initiated by β-secretase cleavage of the amyloid precursor protein (APP). In contrast, soluble APPα (sAPPα) generated by the α-secretase is known to stimulate dendritic branching and enhance synaptic function. Regulation of APP processing, and the shift from neurotrophic to neurotoxic APP metabolism remains poorly understood, but the cellular localization of APP and its interaction with various receptors is considered important. We here identify sortilin as a novel APP interaction partner. Like the related APP receptor SorLA, sortilin is highly expressed in the CNS, but whereas SorLA mainly colocalizes with APP in the soma, sortilin interacts with APP in neurites. The presence of sortilin promotes α-secretase cleavage of APP, unlike SorLA, which inhibits the generation of all soluble products. Also, sortilin and SorLA both bind and mediate internalization of sAPP but to different cellular compartments. The interaction involves the 6A domain of APP, present in both neuronal and non-neuronal APP isoforms. This is important as sAPP receptors described so far only bind the non-neuronal isoforms, leaving SorLA and sortilin as the only receptors for sAPP generated by neurons. Together, our findings establish sortilin, as a novel APP interaction partner that influences both production and cellular uptake of sAPP.

3.1931 Cyclophilin Inhibitors Block Arterivirus Replication by Interfering with Viral RNA Synthesis

De Wilde, A.H., Li, Y., van der Meer, Y., Vuagniaux, G., Lysek, R., Fang, Y., Snijder, E.J. and van Hemert, M.J.
J. Virol., ***87(3)***, *1454-1464 (2013)*

Virus replication strongly depends on cellular factors, in particular, on host proteins. Here we report that the replication of the arteriviruses equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV) is strongly affected by low-micromolar concentrations of cyclosporine A (CsA), an inhibitor of members of the cyclophilin (Cyp) family. In infected cells, the expression of a green fluorescent protein (GFP) reporter gene inserted into the PRRSV genome was inhibited with a half-maximal inhibitory concentration (IC_{50}) of 5.2 μM, whereas the GFP expression of an EAV-GFP reporter virus was inhibited with an IC_{50} of 0.95 μM. Debio-064, a CsA analog that lacks its undesirable immunosuppressive properties, inhibited EAV replication with an IC_{50} that was 3-fold lower than that of CsA, whereas PRRSV-GFP replication was inhibited with an IC_{50} similar to that of CsA. The addition of 4 μM CsA after infection prevented viral RNA and protein synthesis in EAV-infected cells, and CsA treatment resulted in a 2.5- to 4-log-unit reduction of PRRSV or EAV infectious progeny. A complete block of EAV RNA synthesis was also observed in an *in vitro* assay using isolated viral replication structures. The small interfering RNA-mediated knockdown of Cyp family members revealed that EAV replication strongly depends on the expression of CypA but not CypB. Furthermore, upon fractionation of intracellular membranes in density gradients, CypA was found to cosediment with membranous EAV replication structures, which could be prevented by CsA treatment. This suggests that CypA is an essential component of the viral RNA-synthesizing machinery.

3.1932 Lipid Exchange between Borrelia burgdorferi and Host Cells

Crowley, J.T., Toledo, A.M., LaRocca, T.J., Coleman, J.L., London, E. and Benah, J.L.
PloS One, ***9(1)***, *e1003109 (2013)*

Borrelia burgdorferi, the agent of Lyme disease, has cholesterol and cholesterol-glycolipids that are essential for bacterial fitness, are antigenic, and could be important in mediating interactions with cells of the eukaryotic host. We show that the spirochetes can acquire cholesterol from plasma membranes of epithelial cells. In addition, through fluorescent and confocal microscopy combined with biochemical approaches, we demonstrated that *B. burgdorferi* labeled with the fluorescent cholesterol analog BODIPY-cholesterol or ^{3}H-labeled cholesterol transfer both cholesterol and cholesterol-glycolipids to HeLa cells. The transfer occurs through two different mechanisms, by direct contact between the bacteria and eukaryotic cell and/or through release of outer membrane vesicles. Thus, two-way lipid exchange between

spirochetes and host cells can occur. This lipid exchange could be an important process that contributes to the pathogenesis of Lyme disease

3.1933 Modulation of dendritic AMPA receptor mRNA trafficking by RNA splicing and editing
Via, L.L., Bonini, D., Russo, I., Orlandi, C., Barlati, S. and Barbon, A.
Nucleic Acids Research, 41(1), 617-631 (2013)

RNA trafficking to dendrites and local translation are crucial processes for superior neuronal functions. To date, several α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) mRNAs have been detected in dendrites and are subject to local protein synthesis. Here, we report the presence of all AMPAR GluA1-4 mRNAs in hippocampal and cortical rat synaptic spines by synaptoneurosomes analysis. In particular, we showed that dendritic AMPAR mRNAs are present in the Flip versions in the cortex and hippocampus. To further confirm these data, we demonstrate, using in situ hybridization, the dendritic localization of the GluA2 Flip isoform in vitro and in vivo, whereas the Flop variant is restricted mainly to the soma. In addition, we report that dendritic AMPA mRNAs are edited at low levels at their R/G sites; this result was also supported with transfection experiments using chimeric GluA2 DNA vectors, showing that transcripts carrying an unedited nucleotide at the R/G site, in combination with the Flip exon, are more efficiently targeted to dendrites when compared with the edited-Flip versions. Our data show that post-transcriptional regulations such as RNA splicing, editing and trafficking might be mutually coordinated and that the localization of different AMPAR isoforms in dendrites might play a functional role in the regulation of neuronal transmission.

3.1934 Membrane localization of Junín virus glycoproteins requires cholesterol and cholesterol rich membranes
Cordo, S.M., Valko, A., Martinez, G.M. and Candurra, N.A.
*Biochem. Biophys. Res. Comm., **430**, 912-917 (2013)*

Arenavirus morphogenesis and budding occurs at cellular plasma membrane; however, the nature of membrane assembly sites remains poorly understood. In this study we examined the effect of different cholesterol-lowering agents on Junín virus (JUNV) multiplication. We found that cholesterol cell depletion reduced JUNV glycoproteins (GPs) membrane expression and virus budding. Analysis of membrane protein insolubility in Triton X-100 suggested that JUNV GPs associate with cholesterol enriched membranes. Rafts dissociation conditions as warm detergent extraction and cholesterol removal by methyl-β-cyclodextrin compound showed to impair GPs cholesterol enriched membrane association. Analysis of GPs transfected cells showed similar results suggesting that membrane raft association is independent of other viral proteins.

3.1935 The Potential Role of HMGB1 Release in Peritoneal Dialysis-Related Peritonitis
Cao, s., Li, S., Li, H., Xiong, L., Zhou, Y., Fan, J., Yu, X. and Mao, H.
*Plos One, **8(1)**, e54647(2013)*

High mobility group box 1 (HMGB1), a DNA-binding nuclear protein, has been implicated as an endogenous danger signal in the pathogenesis of infection diseases. However, the potential role and source of HMGB1 in the peritoneal dialysis (PD) effluence of patients with peritonitis are unknown. First, to evaluate HMDB1 levels in peritoneal dialysis effluence (PDE), a total of 61 PD patients were enrolled in this study, including 42 patients with peritonitis and 19 without peritonitis. Demographic characteristics, symptoms, physical examination findings and laboratory parameters were recorded. HMGB1 levels in PDE were determined by Western blot and ELISA. The concentrations of TNF-α and IL-6 in PDE were quantified by ELISA. By animal model, inhibition of HMGB1 with glycyrrhizin was performed to determine the effects of HMGB1 in LPS-induced mice peritonitis. *In vitro*, a human peritoneal mesothelial cell line (HMrSV5) was stimulated with lipopolysaccharide (LPS), HMGB1 extracellular content in the culture media and intracellular distribution in various cellular fractions were analyzed by Western blot or immunofluorescence. The results showed that the levels of HMGB1 in PDE were higher in patients with peritonitis than those in controls, and gradually declined during the period of effective antibiotic treatments. Furthermore, the levels of HMGB1 in PDE were positively correlated with white blood cells (WBCs) count, TNF-α and IL-6 levels. However, pretreatment with glycyrrhizin attenuated LPS-induced acute peritoneal inflammation and dysfunction in mice. In cultured HMrSV5 cells, LPS actively induced HMGB1 nuclear-cytoplasmic translocation and release in a time and dose-dependent fashion. Moreover, cytosolic HMGB1 was located in lysosomes and secreted via a lysosome-mediated secretory pathway following LPS stimulation. Our study demonstrates that elevated HMGB1 levels in PDE during PD-related

peritonitis, at least partially, from peritoneal mesothelial cells, which may be involved in the process of PD-related peritonitis and play a critical role in acute peritoneal dysfunction.

3.1936 Critical Role of S1PR1 and Integrin β4 in HGF/c-Met-mediated Increases in Vascular Integrity
Ephstein, Y., Singleton, P.A., Chen, W., Wang, L., Salgia, R., Kanteti, P., Dudek, S.M., Garcia, J.G.N. and Jacobson, J.R.
J. Biol. Chem., ***288(4)***, *2191-2200 (2013)*

Vascular endothelial cell (EC) barrier integrity is critical to vessel homeostasis whereas barrier dysfunction is a key feature of inflammatory disorders and tumor angiogenesis. We previously reported that hepatocyte growth factor (HGF)-mediated increases in EC barrier integrity are signaled through a dynamic complex present in lipid rafts involving its receptor, c-Met (1). We extended these observations to confirm that S1PR1 (sphingosine 1-phosphate receptor 1) and integrin β4 (ITGB4) are essential participants in HGF-induced EC barrier enhancement. Immunoprecipitation experiments demonstrated HGF-mediated recruitment of c-Met, ITGB4 and S1PR1 to caveolin-enriched lipid rafts in human lung EC with direct interactions of c-Met with both S1PR1 and ITGB4 accompanied by c-Met-dependent S1PR1 and ITGB4 transactivation. Reduced S1PR1 expression (siRNA) attenuated both ITGB4 and Rac1 activation as well as c-Met/ITGB4 interaction and resulted in decreased transendothelial electrical resistance. Furthermore, reduced ITGB4 expression attenuated HGF-induced c-Met activation, c-Met/S1PR1 interaction, and effected decreases in S1P- and HGF-induced EC barrier enhancement. Finally, the c-Met inhibitor, XL880, suppressed HGF-induced c-Met activation as well as S1PR1 and ITGB4 transactivation. These results support a critical role for S1PR1 and ITGB4 transactivation as rate-limiting events in the transduction of HGF signals via a dynamic c-Met complex resulting in enhanced EC barrier integrity.

3.1937 Identification of glycosyltransferases involved in cell wall synthesis of wheat endosperm
Suliman, M., Chateigner-Boutin, A.L., Francin-Allami, M., Partier, A., Bouchet, B., Salse, J., Pont, C., Marion, J., Rogniaux, H., Tessier, D., Guillon, F. and Larre, C.
J. Proteomics, ***78***, *508-521 (2013)*

Plant cell walls are complex structures critical for plant fitness and valuable for human nutrition as dietary fiber and for industrial uses such as biofuel production. The cell wall polysaccharides in wheat endosperm consist of two major polymers, arabinoxylans and beta-glucans, as well as other minor components. Most of these polysaccharides are synthesized in the Golgi apparatus but the mechanisms underlying their synthesis have yet to be fully elucidated and only a few of the enzymes involved have been characterized. To identify actors involved in the wheat endosperm cell wall formation, we used a subcellular fractionation strategy to isolate Golgi-enriched fractions from endosperm harvested during active cell wall deposition. The proteins extracted from these Golgi-enriched fractions were analyzed by LC–MS/MS. We report the identification of 1135 proteins among which 64 glycosyltransferases distributed in 17 families. Their potential function in cell wall synthesis is discussed. In addition, we identified 63 glycosylhydrolases, some of which may be involved in cell wall remodeling. Several glycosyltransferases were validated by showing that when expressed as fusion proteins with a fluorescent reporter, they indeed accumulate in the Golgi apparatus. Our results provide new candidates potentially involved in cell wall biogenesis in wheat endosperm.

3.1938 Staphylococcus aureus α-Toxin-Dependent Induction of Host Cell Death by Membrane-Derived Vesicles
Thay, B., Wai, S.N. and Oscarsson, J.
PloS One, ***8(1)***, *e54661 (2013)*

Staphylococcus aureus causes a wide spectrum of infections in humans, ranging from superficial cutaneous infections, infections in the circum-oral region, to life-threatening bacteremia. It was recently demonstrated that Gram-positive organisms such as *S. aureus* liberate membrane-derived vesicles (MVs), which analogously to outer membrane vesicles (OMVs) of Gram-negative bacteria can play a role in delivering virulence factors to host cells. In the present study we have shown that cholesterol-dependent fusion of *S. aureus* MVs with the plasma membrane represents a route for delivery of a key virulence factor, α-toxin (α-hemolysin; Hla) to human cells. Most *S. aureus* strains produce this 33-kDa pore-forming protein, which can lyse a wide range of human cells, and induce apoptosis in T-lymphocytes. Our results revealed a tight association of biologically active α-toxin with membrane-derived vesicles isolated from *S. aureus* strain 8325-4. Concomitantly, α-toxin contributed to HeLa cell cytotoxicity of MVs, and was the main vesicle-associated protein responsible for erythrocyte lysis. In contrast, MVs obtained from

an isogenic *hla* mutant were significantly attenuated with regards to both causing lysis of erythrocytes and death of HeLa cells. This is to our knowledge the first recognition of an *S. aureus* MV-associated factor contributing to host cell cytotoxicity

3.1939 Ataxia with Cerebellar Lesions in Mice Expressing Chimeric PrP-Dpl Protein
Lemaire-Vieille, C., Bailly, Y., Erlich, P., Loeuillet, C., Brocard, J., Haeberle, A.M., Bombarde, G., Rak, C., Demais, V., Dumestre-Perard, C., Gagnon, J. and Cesbron, J-Y.
J. Neurosci., ***33(4)****, 1391-1399 (2013)*

Mutations within the central region of prion protein (PrP) have been shown to be associated with severe neurotoxic activity similar to that observed with Dpl, a PrP-like protein. To further investigate this neurotoxic effect, we generated lines of transgenic (Tg) mice expressing three different chimeric PrP-Dpl proteins. Chi1 (amino acids 1–57 of Dpl replaced by amino acids 1–125 of PrP) and Chi2 (amino acids 1–66 of Dpl replaced by amino acids 1–134 of PrP) abrogated the pathogenicity of Dpl indicating that the presence of a N-terminal domain of PrP (23–134) reduced the toxicity of Dpl, as reported. However, when the amino acids 1–24 of Dpl were replaced by amino acids 1–124 of PrP, Chi3 Tg mice, which express the chimeric protein at a very low level, start developing ataxia at the age of 5–7 weeks. This phenotype was not counteracted by a single copy of full-length-PrP^c but rather by its overexpression, indicating the strong toxicity of the chimeric protein Chi3. Chi3 Tg mice exhibit severe cerebellar atrophy with a significant loss of granule cells. We concluded that aa25 to aa57 of Dpl, which are not present in Chi1 and Chi2 constructs, confer toxicity to the protein. We tested this possibility by using the 25–57 Dpl peptide in primary culture of mouse embryo cortical neurons and found a significant neurotoxic effect. This finding identifies a protein domain that plays a role in mediating Dpl-related toxicity.

3.1940 Acidocalcisomes of Trypanosoma brucei have an inositol 1,4,5-trisphosphate receptor that is required for growth and infectivity
Huang, G., Bartlett, P.J., Thomas, A.P., Moreno, S.N.J. and Docampo, R.
PNAS, ***110(5)****, 1887-1892 (2013)*

Acidocalcisomes are acidic calcium stores rich in polyphosphate and found in a diverse range of organisms. The mechanism of Ca^{2+} release from these organelles was unknown. Here we present evidence that *Trypanosoma brucei* acidocalcisomes possess an inositol 1,4,5-trisphosphate receptor ($TbIP_3R$) for Ca^{2+} release. Localization studies in cell lines expressing *$TbIP_3R$* in its endogenous locus fused to an epitope tag revealed its partial colocalization with the vacuolar proton pyrophosphatase, a marker of acidocalcisomes. IP_3 was able to stimulate Ca^{2+} release from a chicken B-lymphocyte cell line in which the genes for all three vertebrate IP_3Rs have been stably ablated (DT40-3KO) and that were stably expressing *$TbIP_3R$*, providing evidence of its function. IP_3 was also able to release Ca^{2+} from permeabilized trypanosomes or isolated acidocalcisomes and photolytic release of IP_3 in intact trypanosomes loaded with Fluo-4 elicited a transient Ca^{2+} increase in their cytosol. Ablation of *$TbIP_3R$* by RNA interference caused a significant reduction of IP_3-mediated Ca^{2+} release in trypanosomes and resulted in defects in growth in culture and infectivity in mice. Taken together, the data provide evidence of the presence of a functional IP_3R as a Ca^{2+} release channel in acidocalcisomes of trypanosomes and suggest that a Ca^{2+} signaling pathway that involves acidocalcisomes is required for growth and establishment of infection.

3.1941 Osmotic shock-dependent redistribution of diacylglycerol kinase 1 to non-ionic detergent-resistant membrane via pleckstrin homology and C1 domains
Matsutomo, D., Isozaki, T., Sakai, H. and Sakane, F.
J. Biochem., ***153(2)****, 179-190 (2013)*

Diacylglycerol kinase (DGK) participates in regulating the intracellular concentrations of two bioactive lipids, diacylglycerol and phosphatidic acid. DGKη1 is a type II isozyme that contains a pleckstrin homology (PH) domain and a pair of C1 domains at the N-terminus and separated catalytic domains (catalytic subdomain-a and b). We previously reported that DGKη1 expressed in COS-7 cells is translocated from the cytoplasm to punctate granules that partially include endosomes in response to stress stimuli such as osmotic shock. However, the biochemical properties of the stress-dependent behaviour of DGKη1 remain unknown. Here, we have found that DGKη1 is redistributed from the cytosol to the non-ionic detergent (Nonidet P-40)-resistant membrane (DRM) in response to osmotic shock. Our results strongly suggested that the Nonidet P-40 insolubility of DGKη1 is due to neither cytoskeleton localization nor lipid raft association, implying that DGKη1 is distributed to detergent-resistant membrane

microdomains that have a low lipid-to-protein ratio. We revealed, using a series of DGKη1 deletion mutants, that the PH and C1 domains play a pivotal role in osmotic shock-dependent DRM redistribution, whereas catalytic subdomain-a negatively regulates the event.

3.1942 Pex11α deficiency impairs peroxisome elongation and division and contributes to nonalcoholic fatty liver in mice

Weng, H., Ji, X., Naito, Y., Endo, K., Ma, X., Takahashi, R., Shen, C., Hirokawa, G., Fukushima, Y. and Iwai, N.

Am. J. Physiol. Endocrinol. Metab., ***304***, *E187-E196 (2013)*

Hepatic triglyceride (TG) accumulation is considered to be a prerequisite for developing nonalcoholic fatty liver (NAFL). Peroxisomes have many important functions in lipid metabolism, including fatty acid β-oxidization. However, the pathogenic link between NAFL and peroxisome biogenesis remains unclear. To examine the molecular and physiological functions of the *Pex11α* gene, we disrupted this gene in mice. Body weights and hepatic TG concentrations in *Pex11α*$^{-/-}$ mice were significantly higher than those in wild-type (WT) mice fed a normal or a high-fat diet. Hepatic TG concentrations in fasted *Pex11α*$^{-/-}$ mice were significantly higher than those in fasted WT mice. Plasma TG levels increased at lower rates in *Pex11α*$^{-/-}$ mice than in WT mice after treatment with the lipoprotein lipase inhibitor tyloxapol. The number of peroxisomes was lower in the livers of *Pex11α*$^{-/-}$ mice than in those of WT mice. Ultrastructural analysis showed that small and regular spherically shaped peroxisomes were more prevalent in *Pex11α*$^{-/-}$ mice fed normal chow supplemented without or with fenofibrate. We observed a significantly higher ratio of empty peroxisomes containing only PMP70, a peroxisome membrane protein, but not catalase, a peroxisome matrix protein, in *Pex11α*$^{-/-}$ mice. The mRNA expression levels of peroxisomal fatty acid oxidation-related genes (ATP-binding cassette, subfamily D, member 2, and acyl-CoA thioesterase 3) were significantly higher in WT mice than those in *Pex11α*$^{-/-}$ mice under fed conditions. Our results demonstrate that Pex11α deficiency impairs peroxisome elongation and abundance and peroxisomal fatty acid oxidation, which contributes to increased lipid accumulation in the liver.

3.1943 JAK2-V617F-mediated signalling is dependent on lipid rafts and statins inhibit JAK2-V617F-dependent cell growth

Griner, L.N., McGraw, K.L., Johnson, J.O., List, A.F. and Reuther, G.W.

Br. J. Hematol., ***160(2)***, *177-187 (2013)*

Aberrant JAK2 signalling plays an important role in the aetiology of myeloproliferative neoplasms (MPNs). JAK2 inhibitors, however, do not readily eliminate neoplastic MPN cells and thus do not induce patient remission. Further understanding JAK2 signalling in MPNs may uncover novel avenues for therapeutic intervention. Recent work has suggested a potential role for cellular cholesterol in the activation of JAK2 by the erythropoietin receptor and in the development of an MPN-like disorder in mice. Our study demonstrates for the first time that the MPN-associated JAK2-V617F kinase localizes to lipid rafts and that JAK2-V617F-dependent signalling is inhibited by lipid raft disrupting agents, which target membrane cholesterol, a critical component of rafts. We also show for the first time that statins, 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, widely used to treat hypercholesterolaemia, induce apoptosis and inhibit JAK2-V617F-dependent cell growth. These cells are more sensitive to statin treatment than non-JAK2-V617F-dependent cells. Importantly, statin treatment inhibited erythropoietin-independent erythroid colony formation of primary cells from MPN patients, but had no effect on erythroid colony formation from healthy individuals. Our study is the first to demonstrate that JAK2-V617F signalling is dependent on lipid rafts and that statins may be effective in a potential therapeutic approach for MPNs.

3.1944 Alterations in ventricular KATP channel properties during aging

Bao, L., Taskin, E., Foster, M., Ray, B., Rosario, R., ananthakrishnan, R., Howlett, S.E., Schmidt, A.M., Ramasamy, R. and Coetzee, W.A.

Aging Cell, ***12(1)***, *167-176 (2013)*

Coronary heart disease remains the principle cause of mortality in the United States. During aging, the efficiency of the cardiovascular system is decreased and the aged heart is less tolerant to ischemic injury. ATP-sensitive K^+ (K_{ATP}) channels protect the myocardium against ischemic damage. We investigated how aging affects cardiac K_{ATP} channels in the Fischer 344 rat model. Expression of K_{ATP} channel subunit mRNA and protein levels was unchanged in hearts from 26-month-old vs. 4-month-old rats. Interestingly, the mRNA expression of several other ion channels (> 80) was also largely unchanged, suggesting that

posttranscriptional regulatory mechanisms occur during aging. The whole-cell K_{ATP} channel current density was strongly diminished in ventricular myocytes from aged male rat hearts (also observed in aged C57BL/6 mouse myocytes). Experiments with isolated patches (inside-out configuration) demonstrated that the K_{ATP} channel unitary conductance was unchanged, but that the inhibitory effect of cytosolic ATP on channel activity was enhanced in the aged heart. The mean patch current was diminished, consistent with the whole-cell data. We incorporated these findings into an empirical model of the K_{ATP} channel and numerically simulated the effects of decreased cytosolic ATP levels on the human action potential. This analysis predicts lesser activation of K_{ATP} channels by metabolic impairment in the aged heart and a diminished action potential shortening. This study provides insights into the changes in K_{ATP} channels during aging and suggests that the protective role of these channels during ischemia is significantly compromised in the aged individual

3.1945 Role of polymerase η in mitochondrial mutagenesis of Saccharomyces cerevisiae
Chatterje, N., Pabla, R. and Siede, W.
Biochem. Biophys. Res. Comm., ***431***, *270-273 (2013)*

DNA polymerase η mostly catalyzes an error-free bypass of the most frequent UV lesions, pyrimidine dimers of the cyclobutane-type. In addition to its nuclear localization, we show here for the first time its mitochondrial localization in budding yeast. In mitochondria, this polymerase improves bypass replication fidelity opposite UV damage as shown in base pair substitution and frameshift assays. For base pair substitutions, polymerase η appears to be related in function and epistatic to DNA polymerase ζ which, however, plays the opposite role in the nucleus.

3.1946 Mechanistic insights of intestinal absorption and renal conservation of folate in chronic alcoholism
Wani, N.A., Thakur, S., Najar, R.A., Nada, R., Khanduja, K.L. and Kaur, J.
Alcohol, ***47***, *121-130 (2013)*

Folate mediated one-carbon metabolism is of fundamental importance for various cellular processes, including DNA synthesis and methylation of biological molecules. Due to the exogenous requirement of folate in mammals, there exists a well developed epithelial folate transport system for regulation of normal folate homeostasis. The intestinal and renal folate uptake is tightly and diversely regulated and disturbances in folate homeostasis like in alcoholism have pathological consequences. The study was sought to delineate the regulatory mechanism of folate uptake in intestine and reabsorption in renal tubular cells that could evaluate insights of malabsorption during alcoholism. The folate transporters PCFT and RFC were found to be associated with lipid rafts of membrane surfaces in intestine and kidney. Importantly, the observed lower intestinal and renal folate uptake was associated with decreased levels of folate transporter viz. PCFT and RFC in lipid rafts of intestinal and renal membrane surfaces. The decreased association of folate transporters in lipid rafts was associated with decreased protein and mRNA levels. In addition, immunohistochemical studies showed that alcoholic conditions deranged that localization of PCFT and RFC. These findings could explain the possible mechanistic insights that may result in folate malabsorption during alcoholism.

3.1947 Production of Outer Membrane Vesicles and Outer Membrane Tubes by Francisella novicida
McCaig, W.D., Koller, A. and Thanassia, D.G.
J. Bacteriol., ***195(6)***, *1120-1132 (2013)*

Francisella spp. are highly infectious and virulent bacteria that cause the zoonotic disease tularemia. Knowledge is lacking for the virulence factors expressed by Francisella and how these factors are secreted and delivered to host cells. Gram-negative bacteria constitutively release outer membrane vesicles (OMV), which may function in the delivery of virulence factors to host cells. We identified growth conditions under which Francisella novicida produces abundant OMV. Purification of the vesicles revealed the presence of tube-shaped vesicles in addition to typical spherical OMV, and examination of whole bacteria revealed the presence of tubes extending out from the bacterial surface. Recently, both prokaryotic and eukaryotic cells have been shown to produce membrane-enclosed projections, termed nanotubes, which appear to function in cell-cell communication and the exchange of molecules. In contrast to these previously characterized structures, the F. novicida tubes are produced in liquid as well as on solid medium and are derived from the OM rather than the cytoplasmic membrane. The production of the OMV and tubes (OMV/T) by F. novicida was coordinately regulated and responsive to both growth medium and growth phase. Proteomic analysis of purified OMV/T identified known Francisella virulence factors among the constituent proteins, suggesting roles for the vesicles in pathogenesis. In support of this,

production of OM tubes by F. novicida was stimulated during infection of macrophages and addition of purified OMV/T to macrophages elicited increased release of proinflammatory cytokines. Finally, vaccination with purified OMV/T protected mice from subsequent challenge with highly lethal doses of F. novicida.

3.1948 New Type of Outer Membrane Vesicle Produced by the Gram-Negative Bacterium Shewanella vesiculosa M7^T: Implications for DNA Content

Perez-Cruz, C., Carrion, O., Delgado, L., Martinez, G., Lopez-Iglesias, C. and Mercade, e.
Appl. Environ. Microbiol., ***79(6)***, *1874-1881 (2013)*

Outer membrane vesicles (OMVs) from Gram-negative bacteria are known to be involved in lateral DNA transfer, but the presence of DNA in these vesicles has remained difficult to explain. An ultrastructural study of the Antarctic psychrotolerant bacterium Shewanella vesiculosa M7^T has revealed that this Gram-negative bacterium naturally releases conventional one-bilayer OMVs through a process in which the outer membrane is exfoliated and only the periplasm is entrapped, together with a more complex type of OMV, previously undescribed, which on formation drag along inner membrane and cytoplasmic content and can therefore also entrap DNA. These vesicles, with a double-bilayer structure and containing electron-dense material, were visualized by transmission electron microscopy (TEM) after high-pressure freezing and freeze-substitution (HPF-FS), and their DNA content was fluorometrically quantified as 1.8 ± 0.24 ng DNA/μg OMV protein. The new double-bilayer OMVs were estimated by cryo-TEM to represent 0.1% of total vesicles. The presence of DNA inside the vesicles was confirmed by gold DNA immunolabeling with a specific monoclonal IgM against double-stranded DNA. In addition, a proteomic study of purified membrane vesicles confirmed the presence of plasma membrane and cytoplasmic proteins in OMVs from this strain. Our data demonstrate the existence of a previously unobserved type of double-bilayer OMV in the Gram-negative bacterium Shewanella vesiculosa M7^T that can incorporate DNA, for which we propose the name outer-inner membrane vesicle (O-IMV).

3.1949 Caveolin-1 Regulates Endothelial Adhesion of Lung Cancer Cells via Reactive Oxygen Species-Dependen Mechanism

Chanvorachote, P. and Chunhacha, P.
PloS One, ***8(2)***, *e57466 (2013)*

The knowledge regarding the role of caveolin-1 (Cav-1) protein on endothelium adhesion of cancer cells is unclear. The present study revealed that Cav-1 plays a negative regulatory role on cancer-endothelium interaction. Endogenous Cav-1 was shown to down-regulate during cell detachment and the level of such a protein was conversely associated with tumorendothelial adhesion. Furthermore, the ectopic overexpression of Cav-1 attenuated the ability of the cancer cells to adhere to endothelium while shRNA-mediated Cav-1 knock-down exhibited the opposite effect. We found that cell detachment increased cellular hydrogen peroxide and hydroxyl radical generation and such reactive oxygen species (ROS) were responsible for the increasing interaction between cancer cells and endothelial cells through vascular endothelial cell adhesion molecule-1 (VCAM-1). Importantly, Cav-1 was shown to suppress hydrogen peroxide and hydroxyl radical
formation by sustaining the level of activated Akt which was critical for the role of Cav-1 in attenuating the cell adhesion. Together, the present study revealed the novel role of Cav-1 and underlying mechanism on tumor adhesion which explain and highlight an important role of Cav-1 on lung cancer cell metastasis.

3.1950 A Bicyclic 1-Deoxygalactonojirimycin Derivative as a Novel Pharmacological Chaperone for GM_1 Gangliosidosis

Takai, T., Higaki,, K., Aguilar-Moncayo, M., mena-Barragan, T., Hirano, Y., Yura, K., Yu, L., Ninomiiya, H., Garcia-Moreno, M.I., Sakakibara, Y., Ohno, K., Nanba, E., Mellet, C.O., Garcia-Fernandez, J.M. and Suzuki, Y.
Molecular Therapy, ***21(3)***, *526-532 (2013)*

Lysosomal β-galactosidase (β-Gal) deficiency causes a group of disorders that include neuronopathic GM_1 gangliosidosis and non-neuronopathic Morquio B disease. We have previously proposed the use of small molecule ligands of β-Gal as pharmacological chaperones (PCs) for the treatment of GM_1 gangliosidosis brain pathology. Although it is still under development, PC therapy has yielded promising preclinical results in several lysosomal diseases. In this study, we evaluated the effect of bicyclic 1-deoxygalactonojirimycin (DGJ) derivative of the sp^2-iminosugar type, namely 5*N*,6*S*-(*N*′-butyliminomethylidene)-6-thio-1- deoxygalactonojirimycin (6S-NBI-DGJ), as a novel PC for human

mutant β-Gal. *In vitro*, 6S-NBI-DGJ had the ability to inhibit the activity of human β-Gal in a competitive manner and was able to protect this enzyme from heat-induced degradation. Computational analysis supported that the rigid glycone bicyclic core of 6S-NBI-DGJ binds to the active site of the enzyme, with the aglycone *N*′-butyl substituent, in a precise *E*-orientation, located at a hydrophobic region nearby. Chaperone potential profiling indicated significant increases of enzyme activity in 24 of 88 β-Gal mutants, including four common mutations. Finally, oral administration of 6S-NBI-DGJ ameliorated the brain pathology of GM_1 gangliosidosis model mice. These results suggest that 6S-NBI-DGJ is a novel PC that may be effective on a broad range of β-Gal mutants.

3.1951 Sequences within RNA coding for HIV-1 Gag p17 are efficiently targeted to Exosomes
Cabezas, S.C. and Federico, M.
Cell. Microbiol., ***15(3)***, *412-429 (2013)*

HIV budding requires the interaction with cell factors involved in the biogenesis of exosomes. This implies the possibility that viral products undergo exosome incorporation. While this has been already described for both Gag and Nef HIV-1 proteins, no conclusive results on HIV genome have been produced so far. Here, we report that unspliced, but not single or double spliced, HIV-1 RNA species are incorporated in exosomes. Deletion mutant analysis indicated that the presence of a stretch of sequences within the 5′ end of the Gag p17 open reading frame is sufficient for HIV-1 RNA exosome incorporation. These sequences were found associating with exosomes also out of the HIV-1 context, thus indicating that the diversion towards the vesicular compartment can occur without need of additional HIV-1 sequences. Finally, the incorporation of genomic HIV-1 RNA in exosomes significantly increased when producer cells express HIV-1 defective for viral genome packaging. Manipulating infected cells to favour the selective incorporation in exosomes of genomic HIV-1 RNA might have therapeutic implications.

3.1952 Visceral Adipose Tissue-derived Serine Proteinase Inhibitor Inhibits Apoptosis of Endothelial Cells as a Ligand for the Cell-Surface GRP78/Voltage-dependent Anion Channel Complex
Nakatsuka, A., Wada, J., Iseda, I., Teshigawara, S., Higashio, K., Murakami, K., Kanzaki, M., Inoue, K., Terami, T., Katayama, A., Hida, K., Eguchi, J., Ogawa, D., Matsuki, Y., Hiramatsu, R., Yagita, H., Kakuta, S., Iwakura, Y. and Makino, H.
Circ. Res., ***112(5)***, *771-780 (2013)*

Rationale: Visceral adipose tissue-derived serine proteinase inhibitor (vaspin) is an adipokine identified from visceral adipose tissues of genetically obese rats.
Objective: The role of vaspin in the diabetic vascular complications remains elusive, and we investigated the effects of vaspin on the vascular function under the diabetic milieu.
Methods and Results: Adenovirus carrying the full length of the vaspin gene (Vaspin-Ad) ameliorated intimal proliferation of balloon-injured carotid arteries in diabetic Wistar rats. The expression of Ccl2, Pdgfb, and Pdgfrb genes was significantly reduced by the treatment of Vaspin-Ad. In cuff-injured femoral arteries, the intimal proliferation was ameliorated in vaspin transgenic (Vaspin Tg) mice. The application of recombinant vaspin and Vaspin-Ad promoted the proliferation and inhibited the apoptosis of human aortic endothelial cells. Adenovirus expressing vaspin with calmodulin and streptavidin-binding peptides was applied to human aortic endothelial cells, subjected to tandem tag purification and liquid chromatography-tandem mass spectrometry, and we identified GRP78 (78-kDa glucose-regulated protein) as an interacting molecule. The complex formation of vaspin, GRP78, and voltage-dependent anion channel on the plasma membrane was confirmed by the immunoprecipitation studies using aortas of Vaspin Tg mice. The binding assay using ^{125}I-vaspin in human aortic endothelial cells revealed high-affinity binding (dissociation constant = 0.565×10^{-9} m) by the treatment of 5 μM thapsigargin, which recruited GRP78 from the endoplasmic reticulum to plasma membrane by inducing endoplasmic reticulum stress. In human aortic endothelial cells, vaspin induced phosphorylation of Akt and inhibited the kringle 5-induced Ca^{2+} influx and subsequent apoptosis.
Conclusions: Vaspin is a novel ligand for the cell-surface GRP78/voltage-dependent anion channel complex in endothelial cells and promotes proliferation, inhibits apoptosis, and protects vascular injuries in diabetes mellitus.

3.1953 Ablation of very long acyl chain sphingolipids causes hepatic insulin resistance in mice due to altered detergent-resistant membranes
Park, J-W., Park, W-J., Kuperman, Y., Boura-Halfon, S., Pewzner-Jung, Y. and Futerman, A.H.
Hepatology, ***57(2)***, *525-532 (2013)*

Sphingolipids are important structural components of cell membranes and act as critical regulators of cell function by modulating intracellular signaling pathways. Specific sphingolipids, such as ceramide, glucosylceramide, and ganglioside GM3, have been implicated in various aspects of insulin resistance, because they have been shown to modify several steps in the insulin signaling pathway, such as phosphorylation of either protein kinase B (Akt) or of the insulin receptor. We now explore the role of the ceramide acyl chain length in insulin signaling by using a ceramide synthase 2 (CerS2) null mouse, which is unable to synthesize very long acyl chain (C22-C24) ceramides. CerS2 null mice exhibited glucose intolerance despite normal insulin secretion from the pancreas. Both insulin receptor and Akt phosphorylation were abrogated in liver, but not in adipose tissue or in skeletal muscle. The lack of insulin receptor phosphorylation in liver correlated with its inability to translocate into detergent-resistant membranes (DRMs). Moreover, DRMs in CerS2 null mice displayed properties significantly different from those in wild-type mice, suggesting that the altered sphingolipid acyl chain length directly affects insulin receptor translocation and subsequent signaling. *Conclusion:* We conclude that the sphingolipid acyl chain composition of liver regulates insulin signaling by modifying insulin receptor translocation into membrane microdomains

3.1954 Sex-specific response of rat costochondral cartilage growth plate chondrocytes to 17β-estradiol involves differential regulation of plasma membrane associated estrogen receptors
Elbaradie, K.B:Y., Wang, Y., Boyan, B.D. and Schwartz, Z.
*Biochim. Biophys. Acta, **1833**, 1165-1172 (2013)*

Both male and female rat growth plate chondrocytes express estrogen receptors (ERs); however 17 β -estradiol (E_2) induces membrane responses leading to activation of phospholipase A_2 (PLA_2), phospholipase C (PLC), prostaglandin E_2 (PGE_2) production, protein kinase C (PKC), and ultimately mitogen protein kinase (MAPK) only in female cells. This study investigated if these sex-specific responses are due to differences in the actual ERs or in downstream signaling. Western blots and flow cytometry of costochondral cartilage resting zone chondrocytes (RCs) showed 2–3 times more ER α in plasma membranes (PMs) from female cells than male cells. Tunicamycin blocked E_2-dependent ER-translocation to the PM, indicating palmitoylation was required. Co-immunoprecipitation showed E_2 induced complex formation between ER isoforms only in female RCs. To examine if the lack of response in PKC and PGE_2 in males is due to differences in signaling, we examined involvement of ERs and the role of PLC and PLA_2. Selective ER α (propylpyrazole triol, PPT) and ER β (diarylproprionitrile, DPN) agonists activated PKC in female RCs only. The PLC inhibitor, U73122 blocked E_2's effect on PKC and the cytosolic PLA_2 inhibitor, AACOCF3 inhibited the effect on PGE_2 in female RCs, confirming involvement of PLC and PLA_2 in the mechanism. The PLC activator, *m*-3M3F β S activated PKC and PLAA peptide increased PGE_2 levels in male and female RCs, showing that the signaling pathways are present. These data indicate that differences in membrane ER amount, localization, translocation and interaction are responsible for the sexual dimorphic response to E_2.

3.1955 Protein Kinase Cε Modulates Insulin Receptor Localization and Trafficking in Mouse Embryonic Fibroblasts
Pedersen, D.J., Diakanastasis, B., Stöckli, J. and Schmitz-Peiffer, C.
*PloS One, **8(3)**, e58046 (2013)*

We have previously shown that deletion of protein kinase C epsilon (PKCε) in mice results in protection against glucose intolerance caused by a high fat diet. This was in part due to reduced insulin uptake by hepatocytes and insulin clearance, which enhanced insulin availability. Here we employed mouse embryonic fibroblasts (MEFs) derived from wildtype (WT) and PKCε-deficient ($PKC\varepsilon^{-/-}$) mice to examine this mechanistically. $PKC\varepsilon^{-/-}$ MEFs exhibited reduced insulin uptake which was associated with decreased insulin receptor phosphorylation, while downstream signalling through IRS-1 and Akt was unaffected. Cellular fractionation demonstrated that PKCε deletion changed the localization of the insulin receptor, a greater proportion of which co-fractionated with flotillin-1, a marker of membrane microdomains. Insulin stimulation resulted in redistribution of the receptor in WT cells, while this was markedly reduced in $PKC\varepsilon^{-/-}$ cells. These alterations in insulin receptor trafficking were associated with reduced expression of CEACAM1, a receptor substrate previously shown to modulate insulin clearance. Virally-mediated reconstitution of PKCε in MEFs increased CEACAM1 expression and partly restored the sensitivity of the receptor to insulin-stimulated redistribution. These data indicate that PKCε can affect insulin uptake in MEFs through promotion of receptor-mediated endocytosis, and that this may be mediated by regulation of CEACAM1 expression.

3.1956 A genomic toolkit to investigate kinesin and myosin motor function in cells

Maliga, Z., Junqueira, M., Toyoda, Y., Ettinger, A., Mora-Bermudez, F., Klemm, R.W., Vasilj, A., Guhr, E., Ibarlucea-Benitzer, I., Poser, I., Bonifacio, E., Huttner, W., Shevchenko, A. and Hyman, A.A.
Nature Cell Biol., ***15(3)***, *325-334 (2013)*

Coordination of multiple kinesin and myosin motors is required for intracellular transport, cell motility and mitosis. However, comprehensive resources that allow systems analysis of the localization and interplay between motors in living cells do not exist. Here, we generated a library of 243 amino- and carboxy-terminally tagged mouse and human bacterial artificial chromosome transgenes to establish 227 stably transfected HeLa cell lines, 15 mouse embryonic stem cell lines and 1 transgenic mouse line. The cells were characterized by expression and localization analyses and further investigated by affinity-purification mass spectrometry, identifying 191 candidate protein–protein interactions. We illustrate the power of this resource in two ways. First, by characterizing a network of interactions that targets CEP170 to centrosomes, and second, by showing that kinesin light-chain heterodimers bind conventional kinesin in cells. Our work provides a set of validated resources and candidate molecular pathways to investigate motor protein function across cell lineages.

3.1957 Cross-talk between EGFR and T-cadherin: EGFR activation promotes T-cadherin localization to intercellular contacts

Kyriakakis, E., Maslova, K., Frachet, A., Ferri, N., Contini, A., Pfaff, D., Erne, P., Resink, T.J. and Philippova, M.
Cellular Signalling, ***25***, *1044-1053 (2013)*

Reciprocal cross-talk between receptor tyrosine kinases (RTKs) and classical cadherins (e.g. EGFR/E-cadherin, VEGFR/VE-cadherin) has gained appreciation as a combinatorial molecular mechanism enabling diversification of the signalling environment and according differential cellular responses. Atypical glycosylphosphatidylinositol (GPI)-anchored T-cadherin (T-cad) was recently demonstrated to function as a negative auxiliary regulator of EGFR pathway activation in A431 squamous cell carcinoma (SCC) cells. Here we investigate the reciprocal impact of EGFR activation on T-cad. In resting A431 T-cad was distributed globally over the cell body. Following EGF stimulation T-cad was redistributed to the sites of cell‑cell contact where it colocalized with phosphorylated $EGFR^{Tyr1068}$. T-cad redistribution was not affected by endomembrane protein trafficking inhibitor brefeldin A or *de novo* protein synthesis inhibitor cycloheximide, supporting mobilization of plasma membrane associated T-cad. EGF-induced relocalization of T-cad to cell‑cell contacts could be abrogated by specific inhibitors of EGFR tyrosine kinase activity (gefitinib or lapatinib), lipid raft integrity (filipin), actin microfilament polymerization (cytochalasin D or cytochalasin B), p38MAPK (SB203580) or Rac1 (compound**4**). Erk1/2 inhibitor PD98059 increased phospho-$EGFR^{tyr1068}$ levels and not only amplified effects of EGF but also per se promoted some relocalization of T-cad to cell‑cell contacts. Rac1 activation by EGF was inhibited by gefitinib, lapatinib or SB203580 but amplified by PD98059. Taken together our data suggest that T-cad translocation to cell‑cell contacts is sensitive to the activity status of EGFR, requires lipid raft domain integrity and actin filament polymerization, and crucial intracellular signalling mediators include Rac1 and p38MAPK. The study has revealed a novel aspect of reciprocal cross-talk between EGFR and T-cad.

3.1958 Proteomics in colorectal cancer translational research: Biomarker discovery for clinical applications

De Wit, M., Fineman, R.J.A., Verheul, H.M.W., Meijer, G.A. and Jimenez, C.R
Clin. Biochem., ***46***, *466-479 (2013)*

Colorectal cancer (CRC) is a major cause of cancer-related death in the western world. Screening to detect the disease in an early stage is the most effective approach to tackle this problem. In addition, better diagnostic tools for assessment of prognosis and prediction of response to drug therapy will allow for personalized therapies and better outcomes. Protein biomarkers that reflect tumor biology have the potential to address a wide range of clinical needs. These include diagnostic (screening) biomarkers for early detection, prognostic biomarkers for estimation of disease outcome, predictive biomarkers for adjuvant treatment stratification, and surveillance biomarkers for disease monitoring and treatment response. An important source for the discovery of potential biomarkers comes from mass spectrometry based proteomics research of the biology of CRC development.
Here, we review recent colon cancer proteomics studies directed at identification of biomarker proteins. These include studies that use preclinical models (*i.e.* cell lines or murine tissues) as well as clinical materials (*e.g.* tissue and stool samples). We separately highlight some studies that focused on

identification of cancer stem cell (CSC) related proteins in tumor spheroids, an *in vitro* model system for investigating CRC treatment response.
Recent proteomics studies have generated many new candidate protein biomarkers. However, the lack of follow-up studies that lead to biomarker verification and/or validation remains a limiting factor in the translation of these candidate biomarkers into clinical applications. This is partly due to technological limitations which are bound to diminish with new technologies, including selected reaction monitoring mass spectrometry (SRM-MS). Antibodies are still required, though, both to perform high-throughput validation as well as to develop cost-effective tests for routine use in a clinical setting.

3.1959 Role of Us9 Phosphorylation in Axonal Sorting and Anterograde Transport of Pseudorabies Virus
Kratchmarov, R., Taylor, M.P. and Enquist, L.W.
*PloS One, **8(3)**, e58776 (2013)*

Alphaherpes viruses, such as pseudorabies virus (PRV), undergo anterograde transport in neuronal axons to facilitate anterograde spread within hosts. Axonal sorting and anterograde transport of virions is dependent on the viral membrane protein Us9, which interacts with the host motor protein Kif1A to direct transport. Us9-Kif1A interactions are necessary but not sufficient for these processes, indicating that additional cofactors or post-translational modifications are needed. In this study, we characterized two conserved serine phosphorylation sites (S51 and S53) in the PRV Us9 protein that are necessary for anterograde spread *in vivo*. We assessed the subcellular localization of phospho-Us9 subspecies during infection of neurons and found that the phospho-form is detectable on the majority, but not all, of axonal vesicles containing Us9 protein. In biochemical assays, phospho-Us9 was enriched in lipid raft membrane microdomains, though Us9 phosphorylation did not require prior lipid raft association. During infections of chambered neuronal cultures, we observed only a modest reduction in anterograde spread capacity for diserine mutant Us9, and no defect for monoserine mutants. Conversely, mutation of the kinase recognition sequence residues adjacent to the phosphorylation sites completely abrogated anterograde spread. In live-cell imaging analyses, anterograde transport of virions was reduced during infection with a recombinant PRV strain expressing GFP-tagged diserine mutant Us9. Phosphorylation was not required for Us9-Kif1A interaction, suggesting that Us9-Kif1A binding is a distinct step from the activation and/or stabilization of the transport complex. Taken together, our findings indicate that, while not essential, Us9 phosphorylation enhances Us9-Kif1A-based transport of virions in axons to modulate the overall efficiency of long-distance anterograde spread of infection.

3.1960 Methods for the Study of Dopamine Receptors Within Lipid Rafts of Kidney Cells
Yu, P., Villar, V.A. and Jose, P.A.
*Methods in Mol. Biol., **964**, 15-23 (2013)*

There is increasing evidence that G protein-coupled receptor (GPCR) signaling is regulated in lipid raft microdomains. GPCRs and GPCR-signaling molecules, including G proteins and protein kinases, have been reported to compartmentalize in these microdomains. Dopamine D_1-like receptors (D_1R and D_5R) belong to a family of GPCRs that are important in the regulation of renal function. These receptors are not only localized and regulated in caveolae that contains caveolin-1 but are also distributed in non-caveolar lipid rafts which do not contain caveolin-1. This chapter describes detergent- and non-detergent-based methods to obtain lipid raft fractions from renal proximal tubule cells.

3.1961 Quantitative and Qualitative Preparations of Bacterial Outer Membrane Vesicles
Chutkan, H., MacDonald, I., Manning, A. and Kuehn, M.J.
*Methods in Mol. Biol., **966**, 259-271 (2013)*

Gram-negative bacterial outer membrane vesicle production and function have been studied using a variety of quantitative and qualitative methods. These types of analyses can be hampered by the use of impure vesicle preparations. Here we describe a set of techniques that are useful for the quantitative analysis of vesicle production and for preparative yields of highly purified vesicles for studies of vesicle function or composition. Procedures and advice are also included for the purification of vesicles from encapsulated and low-yield strains.

3.1962 Isolation of Pathogen-Containing Vacuoles
Shechuk, O. and Steinert, M.
*Methods in Mol. Biol., **983**, 419-429 (2013)*

Dictyostelium discoideum cells are “professional phagocytes,” as they ingest a large variety of bacteria, yeast, and inert particles. Several bacterial pathogens are able to survive intracellularly within specialized vacuoles of *D. discoideum* by interfering with host signaling pathways. To better understand the molecular mechanisms underlying these evolutionary conserved processes we have established a method for the isolation of pathogen-containing vacuoles (PCVs). The isolation protocol describes the infection of *D. discoideum* cells with the intracellular pathogen *Legionella pneumophila*, loading of the lysosomal compartment with colloidal iron, mechanical lysis of host cells, iodophenylnitrophenyltetrazolium (INT) heavy labeling of mitochondria, removal of nucleic acid by Benzonase treatment, separation of nuclei by low-speed centrifugation, and the magnetic removal of lysosomes. The subcellular fractionation in a discontinuous sucrose density OptiPrep gradient allows the separation of mitochondria and to prepare PCVs with high purity. The proteins isolated from PCVs have been successfully subjected to mass spectrometry and allowed to analyze pathogen-directed maturation processes of vacuoles. The method can also be applied for subsequent protein modification analyses and lipidome comparisons.

3.1963 Decreased activity of folate transporters in lipid rafts resulted in reduced hepatic folate uptake in chronic alcoholism in rats

Wani, N.A., Nada, R., Khanduja, K.L. and Kaur, J.
Genes Nutr., ***8****, 209-219 (2013)*

Folic acid is an essential nutrient that is required for one-carbon biosynthetic processes and for methylation of biomolecules. Deficiency of this micronutrient leads to disturbances in normal physiology of cell. Chronic alcoholism is well known to be associated with folate deficiency, which is due in part to folate malabsorption. The present study deals with the regulatory mechanisms of folate uptake in liver during chronic alcoholism. Male Wistar rats were fed 1 g/kg body weight/day ethanol (20 % solution) orally for 3 months, and the molecular mechanisms of folate uptake were studied in liver. The characterization of the folate transport system in liver basolateral membrane (BLM) suggested it to be a carrier mediated and acidic pH dependent, with the major involvement of proton coupled folate transporter and folate binding protein in the uptake. The folate transporters were found to be associated with lipid raft microdomain of liver BLM. Moreover, ethanol ingestion decreased the folate transport by altering the *V*max of folate transport process and downregulated the expression of folate transporters in lipid rafts. The decreased transporter levels were associated with reduced protein and mRNA levels of these transporters in liver. The deranged folate uptake together with reduced folate transporter levels in lipid rafts resulted in reduced folate levels in liver and thereby to its reduced levels in serum of ethanol-fed rats. The chronic ethanol ingestion led to decreased folate uptake in liver, which was associated with the decreased number of transporter molecules in the lipid rafts that can be ascribed to the reduced synthesis of these transporters.

3.1964 Facing glycosphingolipid–Shiga toxin interaction: dire straits for endothelial cells of the human vasculature

Bauwens, A., Betz, J., Meisen,I., Kemper, B., Karch, H. and Müthing, J.
Cell. Mol. Life Sci., ***70****, 425-457 (2013)*

The two major Shiga toxin (Stx) types, Stx1 and Stx2, produced by enterohemorrhagic *Escherichia coli* (EHEC) in particular injure renal and cerebral microvascular endothelial cells after transfer from the human intestine into the circulation. Stxs are AB_5 toxins composed of an enzymatically active A subunit and the pentameric B subunit, which preferentially binds to the glycosphingolipid globotriaosylceramide (Gb3Cer/CD77). This review summarizes the current knowledge on Stx-caused cellular injury and the structural diversity of Stx receptors as well as the initial molecular interaction of Stxs with the human endothelium of different vascular beds. The varying lipoforms of Stx receptors and their spatial organization in lipid rafts suggest a central role in different modes of receptor-mediated endocytosis and intracellular destiny of the toxins. The design and development of tailored Stx neutralizers targeting the oligosaccharide–toxin recognition event has become a very real prospect to ameliorate or prevent life-threatening renal and neurological complications.

3.1965 TLR4–MD-2 complex is negatively regulated by an endogenous ligand, globotetraosylceramide

Kondo, Y. et al
PNAS, ***110(12)****, 4714-4719 (2013)*

Although endogenous ligands for Toll-like receptor (TLR)4–myeloid differentiation factor 2 (MD2) have not been well-understood, we here report that a globo-series glycosphingolipid, globotetraosylceramide (Gb4), attenuates the toxicity of lipopolysaccharides (LPSs) by binding to TLR4–MD-2. Because α1,4-galactosyltransferase (A4galt)-deficient mice lacking globo-series glycosphingolipids showed higher sensitivity to LPS than wild-type mice, we examined mechanisms by which globo-series glycosphingolipids attenuate LPS toxicity. Cultured endothelial cells lacking A4galt showed higher expression of LPS-inducible genes upon LPS treatment. In turn, introduction of A4galt cDNA resulted in the neo expression of Gb4, leading to the reduced expression of LPS-inducible genes. Exogenous Gb4 induced similar effects. As a mechanism for the suppressive effects of Gb4 on LPS signals, specific binding of Gb4 to the LPS receptor TLR4–MD-2 was demonstrated by coprecipitation of Gb4 with recombinant MD-2 and by native PAGE. A docking model also supported these data. Taken together with colocalization of TLR4–MD-2 with Gb4 in lipid rafts after LPS stimulation, it was suggested that Gb4 competes with LPS for binding to TLR4–MD-2. Finally, administration of Gb4 significantly protected mice from LPS-elicited mortality. These results suggest that Gb4 is an endogenous ligand for TLR4–MD-2 and is capable of attenuating LPS toxicity, indicating the possibility for its therapeutic application in endotoxin shock.

3.1966 The myosin motor Myo1c is required for VEGFR2 delivery to the cell surface and for angiogenic signaling

Tiwari, A., Jung, J-J., Inamdar, S.M., Nihalani, D. and Choudhury, A.
Am. J. Physiol. Heart Circ. Physiol., ***304***, *H687-H696 (2013)*

Vascular endothelial growth factor receptor-2 (VEGFR2) is a receptor tyrosine kinase that is expressed in endothelial cells and regulates angiogenic signal transduction under both physiological and pathological conditions. VEGFR2 turnover at the plasma membrane (PM) is regulated by its transport through endocytic and secretory transport pathways. Short-range cargo trafficking along actin filaments is commonly regulated by motor proteins of myosin superfamily. In the current study, performed in primary human endothelial cells, we demonstrate that unconventional myosin 1c (Myo1c; class I family member) regulates the localization of VEGFR2 at the PM. We further demonstrate that the recruitment of VEGFR2 to the PM and its colocalization with Myo1c and caveolin-1 occur in response to VEGF-A (VEGF) stimulation. In addition, VEGF-induced delivery of VEGFR2 to the cell surface requires Myo1c; surface VEGFR2 levels are reduced in the absence of Myo1c and, more importantly, are restored by the overexpression of wild-type but not mutant Myo1c. Subcellular density gradient fractionation revealed that partitioning of VEGFR2 into caveolin-1- and Myo1c-enriched membrane fractions is dependent on VEGF stimulation. Myo1c depletion resulted in increased VEGF-induced VEGFR2 transport to the lysosomes for degradation and was rescued by applying either brefeldin A, which blocks trafficking between the endoplasmic reticulum and the Golgi complex, or dynasore, an inhibitor of dynamin-mediated endocytosis. Myo1c depletion also reduced VEGF-induced VEGFR2 phosphorylation at Y1175 and phosphorylation-dependent activation of ERK1/2 and c-Src kinase, leading to reduced cell proliferation and cell migration. This is the first report demonstrating that Myo1c is an important mediator of VEGF-induced VEGFR2 delivery to the cell surface and plays a role in angiogenic signaling.

3.1967 Enhancing Mitochondrial Calcium Buffering Capacity Reduces Aggregation of Misfolded SOD1 and Motor Neuron Cell Death without Extending Survival in Mouse Models of Inherited Amyotrophic Lateral Sclerosis

Parone, P., Da Cruz, S., Han, J.S., McAlonis-Downes, M., Vetto, A.P., Lee, S.K., Tseng, E. and Cleveland, D.W.
J. Neurosci., ***33(11)***, *4657-4671 (2013)*

Mitochondria have been proposed as targets for toxicity in amyotrophic lateral sclerosis (ALS), a progressive, fatal adult-onset neurodegenerative disorder characterized by the selective loss of motor neurons. A decrease in the capacity of spinal cord mitochondria to buffer calcium (Ca^{2+}) has been observed in mice expressing ALS-linked mutants of SOD1 that develop motor neuron disease with many of the key pathological hallmarks seen in ALS patients. In mice expressing three different ALS-causing SOD1 mutants, we now test the contribution of the loss of mitochondrial Ca^{2+}-buffering capacity to disease mechanism(s) by eliminating ubiquitous expression of cyclophilin D, a critical regulator of Ca^{2+}-mediated opening of the mitochondrial permeability transition pore that determines mitochondrial Ca^{2+} content. A chronic increase in mitochondrial buffering of Ca^{2+} in the absence of cyclophilin D was maintained throughout disease course and was associated with improved mitochondrial ATP synthesis, reduced mitochondrial swelling, and retention of normal morphology. This was accompanied by an attenuation of

glial activation, reduction in levels of misfolded SOD1 aggregates in the spinal cord, and a significant suppression of motor neuron death throughout disease. Despite this, muscle denervation, motor axon degeneration, and disease progression and survival were unaffected, thereby eliminating mutant SOD1-mediated loss of mitochondrial Ca^{2+} buffering capacity, altered mitochondrial morphology, motor neuron death, and misfolded SOD1 aggregates, as primary contributors to disease mechanism for fatal paralysis in these models of familial ALS.

3.1968 Lipid Peroxidation Product 4-Hydroxy-2-Nonenal Promotes Seeding-Capable Oligomer Formation and Cell-to-Cell Transfer of α-Synuclein
Bae, E-J., Ho, D-H., Park, E., Jung, J.W., Cho, K., Hong, J.H., Lee, H-J., Kim, K.P. and Lee, S-J.
Antioxidants & Redox Signaling, ***18(7)****, 770-783 (2013)*

Aims: Abnormal accumulation of α-synuclein aggregates is one of the key pathological features of many neurodegenerative movement disorders and dementias. These pathological aggregates propagate into larger brain regions as the disease progresses, with the associated clinical symptoms becoming increasingly severe and complex. However, the factors that induce α-synuclein aggregation and spreading of the aggregates remain elusive. Herein, we have evaluated the effects of the major lipid peroxidation byproduct 4-hydroxy-2-nonenal (HNE) on α-synuclein oligomerization and cell-to-cell transmission of this protein. ***Results:*** Incubation with HNE promoted the oligomerization of recombinant human α-synuclein via adduct formation at the lysine and histidine residues. HNE-induced α-synuclein oligomers evidence a little β-sheet structure and are distinct from amyloid fibrils at both conformation and ultrastructure levels. Nevertheless, the HNE-induced oligomers are capable of seeding the amyloidogenesis of monomeric α-synuclein under *in vitro* conditions. When neuronal cells were treated with HNE, both the translocation of α-synuclein into vesicles and the release of this protein from cells were increased. Neuronal cells can internalize HNE-modified α-synuclein oligomers, and HNE treatment increased the cell-to-cell transfer of α-synuclein proteins. ***Innovation and Conclusion:*** These results indicate that HNE induces the oligomerization of α-synuclein through covalent modification and promotes the cell-to-cell transfer of seeding-capable oligomers, thereby contributing to both the initiation and spread of α-synuclein aggregates.

3.1969 Drug Uptake, Lipid Rafts, and Vesicle Trafficking Modulate Resistance to an Anticancer Lysophosphatidylcholine Analogue in Yeast
Cuesta-Marban, A. et al
J. Biol. Chem., ***288(12)****, 8405-8418 (2013)*

The ether-phospholipid edelfosine, a prototype antitumor lipid (ATL), kills yeast cells and selectively kills several cancer cell types. To gain insight into its mechanism of action, we performed chemogenomic screens in the *Saccharomyces cerevisiae* gene-deletion strain collection, identifying edelfosine-resistant mutants. *LEM3, AGP2,* and *DOC1* genes were required for drug uptake. Edelfosine displaced the essential proton pump Pma1p from rafts, inducing its internalization into the vacuole. Additional ATLs, including miltefosine and perifosine, also displaced Pma1p from rafts to the vacuole, suggesting that this process is a major hallmark of ATL cytotoxicity in yeast. Radioactive and synthetic fluorescent edelfosine analogues accumulated in yeast plasma membrane rafts and subsequently the endoplasmic reticulum. Although both edelfosine and Pma1p were initially located at membrane rafts, internalization of the drug toward endoplasmic reticulum and Pma1p to the vacuole followed different routes. Drug internalization was not dependent on endocytosis and was not critical for yeast cytotoxicity. However, mutants affecting endocytosis, vesicle sorting, or trafficking to the vacuole, including the retromer and ESCRT complexes, prevented Pma1p internalization and were edelfosine-resistant. Our data suggest that edelfosine-induced cytotoxicity involves raft reorganization and retromer- and ESCRT-mediated vesicular transport and degradation of essential raft proteins leading to cell death. Cytotoxicity of ATLs is mainly dependent on the changes they induce in plasma membrane raft-located proteins that lead to their internalization and subsequent degradation. Edelfosine toxicity can be circumvented by inactivating genes that then result in the recycling of internalized cell-surface proteins back to the plasma membrane.

3.1970 Alteration of Plasma Membrane Organization by an Anticancer Lysophosphatidylcholine Analogue Induces Intracellular Acidification and Internalization of Plasma Membrane Transporters in Yeast
Czyz, O., Bitew, T., Cuesta-Marban, A., McMaster, C.R., Mollinedo, F. and Zaremberg, V.
J. Biol. Chem., ***288(12)****, 8419-8432 (2013)*

The lysophosphatidylcholine analogue edelfosine is a potent antitumor lipid that targets cellular membranes. The underlying mechanisms leading to cell death remain controversial, although two cellular membranes have emerged as primary targets of edelfosine, the plasma membrane (PM) and the endoplasmic reticulum. In an effort to identify conditions that enhance or prevent the cytotoxic effect of edelfosine, we have conducted genome-wide surveys of edelfosine sensitivity and resistance in *Saccharomyces cerevisiae* presented in this work and the accompanying paper (Cuesta-Marbán, Á., Botet, J., Czyz, O., Cacharro, L. M., Gajate, C., Hornillos, V., Delgado, J., Zhang, H., Amat-Guerri, F., Acuña, A. U., McMaster, C. R., Revuelta, J. L., Zaremberg, V., and Mollinedo, F. (January 23, 2013) *J. Biol. Chem.* 288,), respectively. Our results point to maintenance of pH homeostasis as a major player in modulating susceptibility to edelfosine with the PM proton pump Pma1p playing a main role. We demonstrate that edelfosine alters PM organization and induces intracellular acidification. Significantly, we show that edelfosine selectively reduces lateral segregation of PM proteins like Pma1p and nutrient H^+-symporters inducing their ubiquitination and internalization. The biology associated to the mode of action of edelfosine we have unveiled includes selective modification of lipid raft integrity altering pH homeostasis, which in turn regulates cell growth.

3.1971 Role of C-terminal Membrane-proximal Basic Residues in Cell Surface Trafficking of HIV Coreceptor GPR15 Protein

Okamoto, Y., Bernstein, J.D. and Shikano, S.
J. Biol. Chem., ***288(13)***, *9189-9199 (2013)*

Cell surface density of G protein-coupled receptors (GPCRs) is controlled by dynamic molecular interactions that often involve recognition of the distinct sequence signals on the cargo receptors. We reported previously that the R*X*R-type dibasic motif in the distal C-terminal tail of an HIV coreceptor GPR15 negatively regulates the cell surface expression by mediating the coatomer protein I complex-dependent retrograde transport to the endoplasmic reticulum (ER). Here we demonstrate that another pair of basic residues (Arg^{310}-Arg^{311}) in the membrane-proximal region of the C-terminal tail plays a pivotal role in mediating the anterograde trafficking of GPR15. The Ala mutation of the C-terminal membrane-proximal basic residues (MPBRs) (R310/311A) abolished the *O*-glycosylation and cell surface expression of GPR15. The subcellular fractionation and immunocytochemistry assays indicated that the R310/311A mutant was more localized in the ER but much less in the *trans*-Golgi when compared with the wild-type GPR15, suggesting the positive role of Arg^{310}-Arg^{311} in the ER-to-Golgi transport of GPR15. Sequence analysis on human GPCRs showed that the basic residues are frequent in the membrane-proximal region of the C-terminal tail. Similar to GPR15, mutation of the C-terminal MPBRs resulted in a marked reduction of the cell surface expression in multiple different GPCRs. Our results suggest that the C-terminal MPBRs are critically involved in mediating the anterograde trafficking of a broad range of membrane proteins, including GPCRs.

3.1972 Vps35 loss promotes hyperresorptive osteoclastogenesis and osteoporosis via sustained RANKL signaling

Xia, W-F., Tang, F-L., Xiong, L., Xiong, S., Jung, J-U., Lee, D-H., Li, X-S., Feng, X., Mei, L. and Xiong, W-C.
J. Cell Biol., ***200(6)***, *821-837 (2013)*

Receptor activator of NF-κB (RANK) plays a critical role in osteoclastogenesis, an essential process for the initiation of bone remodeling to maintain healthy bone mass and structure. Although the signaling and function of RANK have been investigated extensively, much less is known about the negative regulatory mechanisms of its signaling. We demonstrate in this paper that RANK trafficking, signaling, and function are regulated by VPS35, a major component of the retromer essential for selective endosome to Golgi retrieval of membrane proteins. VPS35 loss of function altered RANK ligand (RANKL)–induced RANK distribution, enhanced RANKL sensitivity, sustained RANKL signaling, and increased hyperresorptive osteoclast (OC) formation. Hemizygous deletion of the Vps35 gene in mice promoted hyperresorptive osteoclastogenesis, decreased bone formation, and caused a subsequent osteoporotic deficit, including decreased trabecular bone volumes and reduced trabecular thickness and density in long bones. These results indicate that VPS35 critically deregulates RANK signaling, thus restraining increased formation of hyperresorptive OCs and preventing osteoporotic deficits.

3.1973 Electrophilic nitro-fatty acids inhibit vascular inflammation by disrupting LPS-dependent TLR4 signalling in lipid rafts

Villacorta, L., Chang, L., Salvatore, S.R., Ichikawa, T., Zhang, J., Petrovic-Djergovic, D., Jia, L., Carlsen, H., Schopfer, F.J., Freeman, B.A. and Chen, Y.E.
Cardiovasc. Res., ***98****, 116-124 (2013)*

Aims Electrophilic fatty acid nitroalkene derivatives, products of unsaturated fatty acid nitration, exert long-term cardiovascular protection in experimental models of metabolic and cardiovascular diseases. The goal of this study is to examine the effects of nitro-fatty acids in the regulation of upstream signalling events in nuclear factor-κB (NF-κB) activation and determine whether low-dose acute administration of nitro-fatty acids reduces vascular inflammation *in vivo*.
Methods and results Using NF-κB-luciferase transgenic mice, it was determined that pre-emptive treatment with nitro-oleic acid (OA-NO_2), but not oleic acid (OA) inhibits lipopolysaccharide (LPS)-induced NF-κB activation both *in vivo* and in isolated macrophages. Acute intravenous administration of OA-NO_2 was equally effective to inhibit leukocyte recruitment to the vascular endothelium assessed by intravital microscopy and significantly reduces aortic expression of adhesion molecules. An acute treatment with OA-NO_2 *in vivo* yielding nanomolar concentrations in plasma, is sufficient to inhibit LPS-induced Toll-like receptor 4 (TLR4)-induced cell surface expression in leukocytes and NF-κB activation. *In vitro* experiments reveal that OA-NO_2 suppresses LPS-induced TLR4 signalling, inhibitor of κB (IκBα) phosphorylation and ubiquitination, phosphorylation of the IκB kinase (IKK), impairing the recruitment of the TLR4 and TNF receptor associated factor 6 (TRAF6) to the lipid rafts compartments.
Conclusion These studies demonstrate that acute administration of nitro-fatty acids is effective to reduce vascular inflammation *in vivo*. These findings reveal a direct role of nitro-fatty acids in the disruption of the TLR4 signalling complex in lipid rafts, upstream events of the NF-κB pathway, leading to resolution of pro-inflammatory activation of NF-κB in the vasculature.

3.1974 CG0009, a Novel Glycogen Synthase Kinase 3 Inhibitor, Induces Cell Death through Cyclin D1 Depletion in Breast Cancer Cells

Kim, H.M., Kim, C-S., Lee, J-H., Jang, S.J., Hwang, J.J., Ro, S. and Choi, J.
PloS One, ***8(4)****, e60383 (2013)*

Glycogen synthase kinase 3α/β (GSK3α/β) is a constitutively active serine/threonine kinase involved in multiple physiological processes, such as protein synthesis, stem cell maintenance and apoptosis, and acts as a key suppressor of the Wnt-β-catenin pathway. In the present study, we examined the therapeutic potential of a novel GSK3 inhibitor, CG0009, in the breast cancer cell lines, BT549, HS578T, MDA-MB-231, NCI/ADR-RES, T47D, MCF7 and MDA-MB-435, from the NCI-60 cancer cell line panel. Assessment of cytotoxicity, apoptosis and changes in estrogen-signaling proteins was performed using cell viability assays, Western blotting and quantitative real-time PCR. CG0009 enhanced the inactivating phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 and simultaneously decreased activating phosphorylation of GSK3β at Tyr216, and induced caspase-dependent apoptosis independently of estrogen receptor α (ERα) expression status, which was not observed with the other GSK3 inhibitors examined, including SB216763, kenpaullone and LiCl. CG0009 treatment (1 μmol/L) completely ablated cyclin D1 expression in a time-dependent manner in all the cell lines examined, except T47D. CG0009 alone significantly activated p53, leading to relocation of p53 and Bax to the mitochondria. GSK3 inhibition by CG0009 led to slight upregulation of the β-catenin target genes, c-Jun and c-Myc, but not cyclin D1, indicating that CG0009-mediated cyclin D1 depletion overwhelms the pro-survival signal of β-catenin, resulting in cell death. Our findings suggest that the novel GSK3 inhibitor, CG0009, inhibits breast cancer cell growth through cyclin D1 depletion and p53 activation, and may thus offer an innovative therapeutic approach for breast cancers resistant to hormone-based therapy.

3.1975 SH3 interactome conserves general function over specific form

Xin, X. et al
Mol. Systems Biol., ***9:652*** *(2013)*

Src homology 3 (SH3) domains bind peptides to mediate protein–protein interactions that assemble and regulate dynamic biological processes. We surveyed the repertoire of SH3 binding specificity using peptide phage display in a metazoan, the worm *Caenorhabditis elegans*, and discovered that it structurally mirrors that of the budding yeast *Saccharomyces cerevisiae*. We then mapped the worm SH3 interactome using stringent yeast two-hybrid and compared it with the equivalent map for yeast. We found that the worm SH3 interactome resembles the analogous yeast network because it is significantly enriched for

proteins with roles in endocytosis. Nevertheless, orthologous SH3 domain-mediated interactions are highly rewired. Our results suggest a model of network evolution where general function of the SH3 domain network is conserved over its specific form.

3.1976 Heparanase Regulates Secretion, Composition, and Function of Tumor Cell-derived Exosomes
Thompson, C.A., Purushothaman, A., Ramani, V.C., Vlodavsky, I. and Sanderson, R.D.
J. Biol. Chem., ***288(14)***, *10093-10099 (2013)*

Emerging evidence indicates that exosomes play a key role in tumor-host cross-talk and that exosome secretion, composition, and functional capacity are altered as tumors progress to an aggressive phenotype. However, little is known regarding the mechanisms that regulate these changes. Heparanase is an enzyme whose expression is up-regulated as tumors become more aggressive and is associated with enhanced tumor growth, angiogenesis, and metastasis. We have discovered that in human cancer cells (myeloma, lymphoblastoid, and breast cancer), when expression of heparanase is enhanced or when tumor cells are exposed to exogenous heparanase, exosome secretion is dramatically increased. Heparanase enzyme activity is required for robust enhancement of exosome secretion because enzymatically inactive forms of heparanase, even when present in high amounts, do not dramatically increase exosome secretion. Heparanase also impacts exosome protein cargo as reflected by higher levels of syndecan-1, VEGF, and hepatocyte growth factor in exosomes secreted by heparanase-high expressing cells as compared with heparanase-low expressing cells. In functional assays, exosomes from heparanase-high cells stimulated spreading of tumor cells on fibronectin and invasion of endothelial cells through extracellular matrix better than did exosomes secreted by heparanase-low cells. These studies reveal that heparanase helps drive exosome secretion, alters exosome composition, and facilitates production of exosomes that impact both tumor and host cell behavior, thereby promoting tumor progression.

3.1977 Analysis of the Early Steps of Herpes Simplex Virus 1 Capsid Tegumentation
Henaff, D., Remillard-Labrosse, G., Loret, S. and Lippe, R.
J. Virol., ***87(9)***, *4895-4906 (2013)*

Herpes simplex virus type 1 particles are multilayered structures with a DNA genome surrounded by a capsid, tegument, and envelope. While the protein content of mature virions is known, the sequence of addition of the tegument and the intracellular compartments where this occurs are intensely debated. To probe this process during the initial stages of egress, we used two approaches: an *in vitro* nuclear egress assay, which reconstitutes the exit of nuclear capsids to the cytoplasm, and a classical nuclear capsid sedimentation assay. As anticipated, *in vitro* cytoplasmic capsids did not harbor U_L34, U_L31, or viral glycoproteins but contained U_S3. In agreement with previous findings, both nuclear and *in vitro* capsids were positive for ICP0 and ICP4. Unexpectedly, nuclear C capsids and cytoplasmic capsids produced *in vitro* without any cytosolic viral proteins also scored positive for U_L36 and U_L37. Immunoelectron microscopy confirmed that these tegument proteins were closely associated with nuclear capsids. When cytosolic viral proteins were present in the *in vitro* assay, no additional tegument proteins were detected on the capsids. As previously reported, the tegument was sensitive to high-salt extraction but, surprisingly, was stabilized by exogenous proteins. Finally, some tegument proteins seemed partially lost during egress, while others possibly were added at multiple steps or modified along the way. Overall, an emerging picture hints at the early coating of capsids with up to 5 tegument proteins at the nuclear stage, the shedding of some viral proteins during nuclear egress, and the acquisition of others tegument proteins during reenvelopment.

3.1978 Bile salt-stimulated phospholipid efflux mediated by ABCB4 localized in nonraft membranes
Morita, S-y., Tsuda, T., Horikami, M., Teraoka, R., Kitagawa, S. and Terada, T.
J. Lipid Res., ***54***, *1221-1230 (2013)*

ABCB4 is necessary for the secretion of phospholipids from hepatocytes into bile and for the protection of cell membranes against bile salts. Lipid rafts are plasma membrane microdomains containing high contents of cholesterol and sphingolipids, which are separated by Triton X-100 extraction or OptiPrep gradient centrifugation. In this study, we investigated the relationship between the function of ABCB4 and lipid rafts using mouse canalicular membranes and HEK293 cells stably expressing ABCB4. ABCB4 and ABCB1 were mainly distributed in nonraft membranes. The expression of ABCB4, but not ABCB1, led to significant increases in the phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM) contents in nonraft membranes and further enrichment of SM and cholesterol in raft membranes. The ABCB4-mediated efflux of PC, PE, and SM was significantly stimulated by taurocholate, while the efflux

of PE and SM was much less than that of PC. This ABCB4-mediated efflux was completely abolished by BODIPY-verapamil, which hardly partitioned into raft membranes. In addition, ABCB1 and ABCB4 mediated the efflux of rhodamine 123 and rhodamine 6G from nonraft membranes, which was not affected by taurocholate. We conclude that ABCB4 located in nonrafts, but not in rafts, is predominantly involved in the efflux of phospholipids and other substrates.

3.1979 In vitro correction of disorders of lysosomal transport by microvesicles derived from baculovirus-infected Spodoptera cells

Thoene, J., Goss, T., Witcher, M., Mullet, J., N'Kuli, F., Van Der Smissen, P., Courtoy, P and Hahn, S.H.
*Mol. Genet. Metabolism, **109**, 77-85 (2013)*

Infection of *Spodoptera frugiperda* (Sf9) cells by baculovirus (BV) is well established for transgene expression of soluble proteins, but few correctly folded transmembrane proteins have been so produced. We here report the use of the BV/Sf9 (BVES) method for the expression and transfer, via microvesicles, of the exclusive lysosomal exporters for cystine and sialic acid, human cystinosin and sialin. These proteins and their mRNA are released into the culture medium as very low-density microvesicles (~ 1.05 g/ml), which do not label for lysobisphosphatidic acid. The presence of the human transgene proteins in the vesicles was confirmed by western blotting and confirmed and quantified by mass spectrometry. Addition of vesicles to cultures of human fibroblast lines deficient in either cystinosin or sialin produced a progressive depletion of stored lysosomal cystine or sialic acid, respectively. The depletion effect was slow ($T_{1/2}$ ~ 48 h), saturable (down to ~ 40% of initial after 4 days) and stable (> one week). Surprisingly, BV infection of *Spodoptera* appeared to induce expression and release into microvesicles of the insect orthologue of cystinosin, but not of sialin. We conclude that BVES is an effective method to express and transfer functional transmembrane proteins so as to study their properties in mammalian cells, and has a generic potential for transport protein replacement therapy.

3.1980 Lipid Droplet-Binding Protein TIP47 Regulates Hepatitis C Virus RNA Replication through Interaction with the Viral NS5A Protein

Vogt, D.A., Camus, G., Herker, E., Webster, B.R., Tsou, C-L., Greene, W.C., Yen, T-S.B. and Ott, M.
*PloS Pathogens, **(9(4)**, e1003302 (2013)*

The nonstructural protein NS5A has emerged as a new drug target in antiviral therapies for Hepatitis C Virus (HCV) infection. NS5A is critically involved in viral RNA replication that takes place at newly formed membranes within the endoplasmic reticulum (membranous web) and assists viral assembly in the close vicinity of lipid droplets (LDs). To identify host proteins that interact with NS5A, we performed a yeast two-hybrid screen with the N-terminus of NS5A (amino acids 1–31), a well-studied α-helical domain important for the membrane tethering of NS5A. Our studies identified the LD-associated host protein, Tail-Interacting Protein 47 (TIP47) as a novel NS5A interaction partner. Coimmunoprecipitation experiments in Huh7 hepatoma cells confirmed the interaction of TIP47 with full-length NS5A. shRNA-mediated knockdown of TIP47 caused a more than 10-fold decrease in the propagation of full-length infectious HCV in Huh7.5 hepatoma cells. A similar reduction was observed when TIP47 was knocked down in cells harboring an autonomously replicating HCV RNA (subgenomic replicon), indicating that TIP47 is required for efficient HCV RNA replication. A single point mutation (W9A) in NS5A that disrupts the interaction with TIP47 but preserves proper subcellular localization severely decreased HCV RNA replication. In biochemical membrane flotation assays, TIP47 cofractionated with HCV NS3, NS5A, NS5B proteins, and viral RNA, and together with nonstructural viral proteins was uniquely distributed to lower-density LD-rich membrane fractions in cells actively replicating HCV RNA. Collectively, our data support a model where TIP47—via its interaction with NS5A—serves as a novel cofactor for HCV infection possibly by integrating LD membranes into the membranous web.

3.1981 Nuclear Targeting of Human Cytomegalovirus Large Tegument Protein pUL48 Is Essential for Viral Growth

Brock, I., Krüger, M., Mertens, T. and von Einem, J.
*J. Virol., **87(10)**, 6005-6019 (2013)*

We report the identification of a functional nuclear localization signal (NLS) in the human cytomegalovirus (HCMV) large tegument protein pUL48 that is required for nuclear localization in transfected cells and is essential for viral growth. The NLS was mapped to pUL48 amino acid residues 284 to 302. This sequence contains a bipartite NLS comprising two clusters of basic residues (bC1 and bC2) separated by 9 amino acids. Deletion or mutation of bC1 or mutation of bC2 abrogated the nuclear

localization of full-length pUL48 in transiently expressing cells, thus strongly implying a bipartite character of the NLS. Nuclear localization could be restored by fusion of a functional NLS together with enhanced green fluorescent protein (EGFP) to the N terminus of these mutants. In HCMV-infected cells, pUL48 was found in both nuclear and cytoplasmic fractions, supporting a function of the NLS during virus infection. NLS mutant viruses, generated by markerless bacterial artificial chromosome mutagenesis, were not viable in cell culture, whereas coexpression of pUL48 complemented growth of these mutants. The fusion of a functional NLS to the N terminus of pUL48 in a nonviable NLS mutant virus partially rescued the growth defect. Furthermore, the replacement of the bipartite pUL48 NLS by the monopartite pUL36 NLS of herpes simplex virus 1 supported viral growth to some extent but still revealed a severe defect in focus formation and release of infectious virus particles. Together, these results show that nuclear targeting of pUL48 is mediated by a bipartite NLS whose function is essential for HCMV growth.

3.1982 **The Receptor Attachment Function of Measles Virus Hemagglutinin Can Be Replaced with an Autonomous Protein That Binds Her2/neu While Maintaining Its Fusion-Helper Function**
Rasbach, A., Abel, T., Münch, R.C., Boller, K., Schneider-Schalies, J. and Buchholz, C.J.
J. Virol., ***87(11)***, *6246-6256 (2013)*

Cell entry of enveloped viruses is initiated by attachment to the virus receptor followed by fusion between the virus and host cell membranes. Measles virus (MV) attachment to its receptor is mediated by the hemagglutinin (H), which is thought to produce conformational changes in the membrane fusion protein (F) that trigger insertion of its fusion peptide into the target cell membrane. Here, we uncoupled receptor attachment and the fusion-helper function of H by introducing Y481A, R533A, S548L, and F549S mutations into the viral attachment protein that made it blind to its normal receptors. An artificial receptor attachment protein specific for Her2/*neu* was incorporated into the membranes of pseudotyped lentivirus particles as a separate transmembrane protein along with the F protein. Surprisingly, these particles entered efficiently into Her2/*neu*-positive SK-OV-3 as well as CHO-Her2 cells. Cell entry was independent of endocytosis but strictly dependent on the presence of H. H-specific monoclonal antibodies, as well as a mutation in H interfering with H/F cooperation, blocked cell entry. The particles mediated stable and specific transfer of reporter genes into Her2/*neu*-positive human tumor cells also *in vivo*, while exhibiting improved infectivity and higher titers than Her2/*neu*-targeted vectors displaying the targeting domain on H. Extending the current model of MV cell entry, the data suggest that receptor binding of H is not required for its fusion-helper function but that particle-cell contact in general may be sufficient to induce the conformational changes in the H/F complex and activate membrane fusion.

3.1983 **Separation of actin-dependent and actin-independent lipid rafts**
Klappe, K., Hummel, I. and Kok, J.W.
Anal. Biochem., ***438***, *133-135 (2013)*

Lipid rafts have been isolated on the basis of their resistance to various detergents and more recently by using detergent-free procedures. The actin cytoskeleton is now recognized as a dynamic regulator of lipid raft stability. We carefully analyzed the effects of the cortical actin-disrupting agent latrunculin B on lipid raft markers of both protein and lipid nature and show that two detergent-free membrane subtypes can be isolated and separated from each other on a one-step density gradient combined with pooling of the appropriate gradient fractions. These two subtypes differ in their dependence on the cortical actin cytoskeleton.

3.1984 **Impairment of Mitochondria in Adult Mouse Brain Overexpressing Predominantly Full-Length, N-Terminally Acetylated Human α-Synuclein**
Sarafian, T.A., Ryan, C.M., Souda, P., Masliah, E., Kar, U.K., Vinters, H.V.., Mathern, G.W., Faull, K.F., Whitelegge, J.P. and Watson, J.B.
PloS One, ***8(5)***, *e63557 (2013)*

While most forms of Parkinson's Disease (PD) are sporadic in nature, a small percentage of PD have genetic causes as first described for dominant, single base pair changes as well as duplication and triplication in the α-synuclein gene. The α-synuclein gene encodes a 140 amino acid residue protein that interacts with a variety of organelles including synaptic vesicles, lysosomes, endoplasmic reticulum/Golgi vesicles and, reported more recently, mitochondria. Here we examined the structural and functional interactions of human α-synuclein with brain mitochondria obtained from an early, pre-manifest mouse model for PD over-expressing human α-synuclein (ASOTg). The membrane potential in ASOTg brain mitochondria was decreased relative to wildtype (WT) mitochondria, while reactive oxygen species (ROS)

were elevated in ASOTg brain mitochondria. No selective interaction of human α-synuclein with mitochondrial electron transport complexes cI-cV was detected. Monomeric human α-synuclein plus carboxyl terminally truncated forms were the predominant isoforms detected in ASOTg brain mitochondria by 2-dimensional PAGE (Native/SDS) and immunoblotting. Oligomers or fibrils were not detected with amyloid conformational antibodies. Mass spectrometry of human α-synuclein in both ASOTg brain mitochondria and homogenates from surgically resected human cortex demonstrated that the protein was full-length and postranslationally modified by N-terminal acetylation. Overall the study showed that accumulation of full-length, N-terminally acetylated human α-synuclein was sufficient to disrupt brain mitochondrial function in adult mice.

3.1985 Exosomes for drug delivery — a novel application for the mesenchymal stem cell
Lai, R.C., Yeo, R.W.Y., Tan, K.H. and Lim, S.K.
*Biotechnolgy Advances, **31**, 543-551 (2013)*

Exosomes are the most extensively characterized class of secreted membrane vesicles that carry proteins and RNAs for intercellular communication. They are increasingly seen as possible alternatives to liposomes as drug delivery vehicles. Like liposomes, they could deliver their cargo across the plasma membrane and provide a barrier against premature transformation and elimination. In addition, these naturally-occurring secreted membrane vesicles are less toxic and better tolerated in the body as evidenced by their ubiquitous presence in biological fluids, and have an intrinsic homing ability. They are also amenable to in vivo and in vitro loading of therapeutic agents, and membrane modifications to enhance tissue-specific homing. Here we propose human mesenchymal stem cells as the ideal cell source of exosomes for drug delivery. Mesenchymal stem cell transplantation for various disease indications has been extensively tested and shown to be safe in numerous clinical trials. These cells are also prolific producers of immunologically inert exosomes. Immortalization of these cells does not compromise the quantity or quality of exosome production, thus enabling infinite and reproducible exosome production from a single cell clone.

3.1986 Angiotensin II Impairs Endothelial Nitric-oxide Synthase Bioavailability under Free Cholesterol-enriched Conditions via Intracellular Free Cholesterol-rich Membrane Microdomains
Amiya, E., Watanabe, M., Takeda, N., Saito, T., Shiga, T., Hosoya, Y., Nakao, T., Imai, Y., Manabe, I., Nagai, R., Komuro, I. and Maemura, K.
*J. Biol. Chem., **288(20)**, 14497-14509 (2013)*

Vascular endothelial function is impaired in hypercholesterolemia partly because of injury by modified LDL. In addition to modified LDL, free cholesterol (FC) is thought to play an important role in the development of endothelial dysfunction, although the precise mechanisms remain to be elucidated. The aim of this study was to clarify the mechanisms of endothelial dysfunction induced by an FC-rich environment. Loading cultured human aortic endothelial cells with FC induced the formation of vesicular structures composed of FC-rich membranes. Raft proteins such as phospho-caveolin-1 (Tyr-14) and small GTPase Rac were accumulated toward FC-rich membranes around vesicular structures. In the presence of these vesicles, angiotensin II-induced production of reactive oxygen species (ROS) was considerably enhanced. This ROS shifted endothelial NOS (eNOS) toward vesicle membranes and vesicles with a FC-rich domain trafficked toward perinuclear late endosomes/lysosomes, which resulted in the deterioration of eNOS Ser-1177 phosphorylation and NO production. Angiotensin II-induced ROS decreased the bioavailability of eNOS under the FC-enriched condition.

3.1987 Estradiol accelerates the effects of fluoxetine on serotonin 1A receptor signaling
Li, Q., Sullivan, N.R., McAllister, C.E., Van de Kar, L.D. and Muma, N.A.
*Psychoneuroendocrinology, **38**, 1145-1157 (2013)*

A major problem with current anti-depressant therapy is that it takes on average 6–7 weeks for remission. Since desensitization of serotonin (5-HT)$_{1A}$ receptor signaling contributes to the anti-depressive response, acceleration of the desensitization may reduce this delay in response to antidepressants. The purpose of the present study was to test the hypothesis that estradiol accelerates fluoxetine-induced desensitization of 5-HT$_{1A}$ receptor signaling in the paraventricular nucleus of the hypothalamus (PVN) of rats, via alterations in components of the 5-HT$_{1A}$ receptor signaling pathway. Ovariectomized rats were injected with estradiol and/or fluoxetine, then adrenocorticotropic hormone (ACTH) and oxytocin responses to a 5-HT$_{1A}$ receptor agonist (+)-8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) were examined to assess the function of 5-

HT_{1A} receptors in the PVN. Treatment with estradiol for either 2 or 7 days or fluoxetine for 2 days produced at most a partial desensitization of 5-HT_{1A} receptor signaling, whereas 7 days of fluoxetine produced full desensitization. Combined treatment with estradiol and fluoxetine for 2 days produced nearly a full desensitization, demonstrating an accelerated response compared to either treatment alone. With two days of combined treatments, estradiol prevented the fluoxetine-induced increase in 5-HT_{1A} receptor protein, which could contribute to the more rapid desensitization. Furthermore, EB treatment for 2 days decreased the abundance of the 35 kD G α z protein which could contribute to the desensitization response. We found two isoforms of G α z proteins with molecular mass of 35 and 33 kD, which differentially distributed in the detergent resistant microdomain (DRM) and in Triton X-100 soluble membrane region, respectively. The 35 kD G α z proteins in the DRM can be sumoylated by SUMO1. Stimulation of 5-HT_{1A} receptors with 8-OH-DPAT increases the sumoylation of G α z proteins and reduces the 33 kD G α z proteins, suggesting that these responses may be related to the desensitization of 5-HT_{1A} receptors. Treatment with estradiol for 2 days also reduced the levels of the G-protein coupled estrogen receptor GPR30, possibly limiting to the ability of estradiol to produce only a partial desensitization response. These data provide evidence that estradiol may be effective as a short-term adjuvant to SSRIs to accelerate the onset of therapeutic effects.

3.1988 Rhesus Monkey Rhadinovirus Uses Eph Family Receptors for Entry into B Cells and Endothelial Cells but Not Fibroblasts
Hahn, A.S. and Desrosiers, R.C.
*PloS One, **9(5)**, e1003360 (2013)*

Cellular Ephrin receptor tyrosine kinases (Ephrin receptors, Ephs) were found to interact efficiently with the gH/gL glycoprotein complex of the rhesus monkey rhadinovirus (RRV). Since EphA2 was recently identified as a receptor for the Kaposi's sarcoma-associated herpesvirus (KSHV) (Hahn et al., Nature Medicine 2012), we analyzed RRV and KSHV in parallel with respect to Eph-binding and Eph-dependent entry. Ten of the 14 Eph proteins, including both A- and B-type, interacted with RRV gH/gL. Two RRV strains with markedly different gH/gL sequences exhibited similar but slightly different binding patterns to Ephs. gH/gL of KSHV displayed high affinity towards EphA2 but substantially weaker binding to only a few other Ephs of the A-type. Productive entry of RRV 26-95 into B cells and into endothelial cells was essentially completely dependent upon Ephs since expression of a GFP reporter cassette from recombinant virus could be blocked to greater than 95% by soluble Eph decoys using these cells. In contrast, entry of RRV into fibroblasts and epithelial cells was independent of Ephs by these same criteria. Even high concentrations and mixtures of soluble Eph decoys were not able to reduce by any appreciable extent the number of fibroblasts and epithelial cells productively entered by RRV. Thus, RRV is similar to its close relative KSHV in the use of Eph family receptors for productive entry into B cells and endothelial cells. However, RRV uses a separate, distinct, Eph-independent pathway for productive entry into fibroblasts and epithelial cells. Whether KSHV also uses an Eph-independent pathway in some circumstances or to some extent remains to be determined.

3.1989 Autophagic failure promotes the exocytosis and intercellular transfer of α-synuclein
Lee, H-J., Cho, E-D., Lee, K.W., Kim, J-H., Cho, S-G. and Lee, S-J.
Exp. Mol. Med., 45, e22 (2013)

The accumulation of abnormal protein aggregates is a major characteristic of many neurodegenerative disorders, including Parkinson's disease (PD). The intracytoplasmic deposition of α-synuclein aggregates and Lewy bodies, often found in PD and other α-synucleinopathies, is thought to be linked to inefficient cellular clearance mechanisms, such as the proteasome and autophagy/lysosome pathways. The accumulation of α-synuclein aggregates in neuronal cytoplasm causes numerous autonomous changes in neurons. However, it can also affect the neighboring cells through transcellular transmission of the aggregates. Indeed, a progressive spreading of Lewy pathology among brain regions has been hypothesized from autopsy studies. We tested whether inhibition of the autophagy/lysosome pathway in α-synuclein-expressing cells would increase the secretion of α-synuclein, subsequently affecting the α-synuclein deposition in and viability of neighboring cells. Our results demonstrated that autophagic inhibition, via both pharmacological and genetic methods, led to increased exocytosis of α-synuclein. In a mixed culture of α-synuclein-expressing donor cells with recipient cells, autophagic inhibition resulted in elevated transcellular α-synuclein transmission. This increase in protein transmission coincided with elevated apoptotic cell death in the recipient cells. These results suggest that the inefficient clearance of α-synuclein aggregates, which can be caused by reduced autophagic activity, leads to elevated α-synuclein

exocytosis, thereby promoting α-synuclein deposition and cell death in neighboring neurons. This finding provides a potential link between autophagic dysfunction and the progressive spread of Lewy pathology.

3.1990 Lipid Droplets, Perilipins and Cytokeratins – Unravelled Liaisons in Epithelium-Derived Cells
Heid, H., Rickelt, S., Zimbelmann, R., Winter, S., Schumacher, H. and Dörflinger, Y.
*PloS One, **8(5)**, e63061 (2013)*

Lipid droplets (LDs) are spherical accumulations of apolar lipids and other hydrophobic substances and are generally surrounded by a thin cortical layer of specific amphiphilic proteins (APs). These APs segregate the LDs from the mostly polar components of the cytoplasm. We have studied LDs in epithelium-derived cell cultures and in particular characterized proteins from the perilipin (PLIN) gene family - in mammals consisting of the proteins *Perilipin, Adipophilin, TIP47, S3-12 and MLDP/OXPAT (PLIN 1-5).* Using a large number of newly generated and highly specific mono- and polyclonal antibodies specific for individual APs, and using improved LD isolation methods, we have enriched and characterized APs in greater detail and purity. The majority of lipid-AP complexes could be obtained in the top layer fractions of density gradient centrifugation separations of cultured cells, but APs could also be detected in other fractions within such separations. The differently sized LD complexes were analyzed using various biochemical methods and mass spectrometry as well as immunofluorescence and electron– in particular immunoelectron-microscopy. Moreover, by immunoprecipitation, protein-protein binding assays and by immunoelectron microscopy we identified a direct linkage between LD-binding proteins and the intermediate-sized filaments (IF) cytokeratins 8 and 18 (also designated as keratins K8 and K18). Specifically, in gradient fractions of higher density supposedly containing small LDs, we received as co-precipitations cytidylyl-, palmitoyl- and cholesterol transferases and other specific enzymes involved in lipid metabolism. So far, common proteomic studies have used LDs from top layer fractions only and did not report on these transferases and other enzymes. In addition to findings of short alternating hydrophobic/hydrophilic segments within the PLIN protein family, we propose and discuss a model for the interaction of LD-coating APs with IF proteins.

3.1991 The HtrA protease of Borrelia burgdorferi degrades outer membrane protein BmpD and chemotaxis phosphatase CheX
Coleman, J.L., Crowley, J.T., Toledo, A.M. and Benach, J.L.
*Mol. Microbiol., **88(3)**, 619-633 (2013)*

Borrelia burgdorferi, the spirochaetal agent of Lyme disease, codes for a single HtrA protein, HtrABb (BB0104) that is homologous to DegP of *Escherichia coli* (41% amino acid identity). HtrABb shows physical and biochemical similarities to DegP in that it has the trimer as its fundamental unit and can degrade casein via its catalytic serine. Recombinant HtrABb exhibits proteolytic activity *in vitro*, while a mutant (HtrABbS198A) does not. However, HtrABb and DegP have some important differences as well. Native HtrABb occurs in both membrane-bound and soluble forms. Despite its homology to DegP, HtrABb could not complement an *E. coli* DegP deletion mutant. Late stage Lyme disease patients, as well as infected mice and rabbits developed a robust antibody response to HtrABb, indicating that it is a B-cell antigen. In co-immunoprecipitation studies, a number of potential binding partners for HtrABb were identified, as well as two specific proteolytic substrates, basic membrane protein D (BmpD/BB0385) and chemotaxis signal transduction phosphatase CheX (BB0671). HtrABb may function in regulating outer membrane lipoproteins and in modulating the chemotactic response of *B. burgdorferi*.

3.1992 PICK1 and ICA69 Control Insulin Granule Trafficking and Their Deficiencies Lead to Impaired Glucose Tolerance
Cao, M., Mao, Z., Kam, C., Xiao, N., Cao, X., Shen, C., Cheng, K.K.Y., Xu, A., Lee, K-M., Jiang, L: and Xia, J.
*PloS Biology, **11(4)**, e1001541 (2013)*

Diabetes is a metabolic disorder characterized by hyperglycemia. Insulin, which is secreted by pancreatic beta cells, is recognized as the critical regulator of blood glucose, but the molecular machinery responsible for insulin trafficking remains poorly defined. In particular, the roles of cytosolic factors that govern the formation and maturation of insulin granules are unclear. Here we report that PICK1 and ICA69, two cytosolic lipid-binding proteins, formed heteromeric BAR-domain complexes that associated with insulin granules at different stages of their maturation. PICK1-ICA69 heteromeric complexes associated with

immature secretory granules near the trans-Golgi network (TGN). A brief treatment of Brefeldin A, which blocks vesicle budding from the Golgi, increased the amount of PICK1 and ICA69 at TGN. On the other hand, mature secretory granules were associated with PICK1 only, not ICA69. PICK1 deficiency in mice caused the complete loss of ICA69 and led to increased food and water intake but lower body weight. Glucose tolerance tests demonstrated that these mutant mice had high blood glucose, a consequence of insufficient insulin. Importantly, while the total insulin level was reduced in PICK1-deficient beta cells, proinsulin was increased. Lastly, ICA69 knockout mice also displayed similar phenotype as the mice deficient in PICK1. Together, our results indicate that PICK1 and ICA69 are key regulators of the formation and maturation of insulin granules.

3.1993 Transforming Growth Factor-β1 (TGF-β1)-stimulated Fibroblast to Myofibroblast Differentiation Is Mediated by Hyaluronan (HA)-facilitated Epidermal Growth Factor Receptor (EGFR) and CD44 Co-localization in Lipid Rafts

Midgley, A.C., Rogers, M., Hallett, M.B., Clayton, A., Bowen, T., Phillips, A.O. and Steadman, R.
J. Biol. Chem., ***288(21)***, *14824-14838 (2013)*

Fibroblast to myofibroblast differentiation drives effective wound healing and is largely regulated by the cytokine transforming growth factor-β1 (TGF-β1). Myofibroblasts express α-smooth muscle actin and are present in granulation tissue, where they are responsible for wound contraction. Our previous studies show that fibroblast differentiation in response to TGF-β1 is dependent on and mediated by the linear polysaccharide hyaluronan (HA). Both the HA receptor, CD44, and the epidermal growth factor receptor (EGFR) are involved in this differentiation response. The aim of this study was to understand the mechanisms linking HA-, CD44-, and EGFR-regulated TGF-β1-dependent differentiation. CD44 and EGFR co-localization within membrane-bound lipid rafts was necessary for differentiation, and this triggered downstream mitogen-activated protein kinase (MAPK/ERK) and Ca^{2+}/calmodulin kinase II (CaMKII) activation. We also found that ERK phosphorylation was upstream of CaMKII phosphorylation, that ERK activation was necessary for CaMKII signaling, and that both kinases were essential for differentiation. In addition, HA synthase-2 (HAS2) siRNA attenuated both ERK and CaMKII signaling and sequestration of CD44 into lipid rafts, preventing differentiation. In summary, the data suggest that HAS2-dependent production of HA facilitates TGF-β1-dependent fibroblast differentiation through promoting CD44 interaction with EGFR held within membrane-bound lipid rafts. This induces MAPK/ERK, followed by CaMKII activation, leading to differentiation. This pathway is synergistic with the classical TGF-β1-dependent SMAD-signaling pathway and may provide a novel opportunity for intervention in wound healing.

3.1994 Analysis of subcellular [57Co] cobalamin distribution in SH-SY5Y neurons and brain tissue

Zhao, H., Ruberu, K., Li, H. and garner, B.
J. Neuroscience Methods, ***2217***, *67-74 (2013)*

Cobalamin (Cbl) utilization as a cofactor for methionine synthase and methylmalonyl-CoA mutase is dependent on the transport of Cbl through lysosomes and its subsequent delivery to the cytosol and mitochondria. We speculated that neuropathological conditions that impair lysosomal function (e.g., age-related lipofuscinosis and specific neurodegenerative diseases) might impair lysosomal Cbl transport. To address this question, an appropriate method to quantify intracellular Cbl transport in neuronal cell types and brain tissue is required. Thus, we developed methods to measure [^{57}Co] Cbl levels in lysosomes, mitochondria and cytosol obtained from in vitro and in vivo sources. Human SH-SY5Y neurons or HT1080 fibroblasts were labeled with [^{57}Co] Cbl and homogenized using a ball-bearing homogenizer, and the lysates were separated into 10 fractions using ultracentrifugation in an OptiPrep density gradient. Lysosomes were recovered from the top of the gradient (fractions 1–5), which were clearly separated from mitochondria (fractions 7–9) on the basis of the expression of the marker proteins, LAMP2 and VDAC1. The isolated lysosomes were intact based on their colocalization with acid phosphatase activity. The lysosomal and mitochondrial fractions were free of the cytosolic markers beta-actin and methionine synthase. The relative distribution of [^{57}Co] Cbl in both neurons and fibroblasts was as follows: 6% in the lysosomes, 14% in the mitochondria and 80% in the cytosol. This technique was also used to fractionate organelles from mouse brain, where marker proteins were detected in the gradient at positions similar to those observed for the cell lines, and the relative distribution of [^{57}Co] Cbl was as follows: 12% in the lysosomes, 15% in the mitochondria and 73% in the cytosol. These methods provide a useful tool for the investigation of intracellular Cbl trafficking in a neurobiological setting.

3.1995 Perilipin-Mediated Lipid Droplet Formation in Adipocytes Promotes Sterol Regulatory Element-Binding Protein-1 Processing and Triacylglyceride Accumulation

Takahashi, Y., Shinoda, A., Furuya, N., Harada, E., Arimura, N., Ichi, I., Fujiwara, Y., Inoue, J. and Sato, R.

*PloS One, **8(5)**, e64605 (2013)*

Sterol regulatory element-binding protein-1 (SREBP-1) has been thought to be a critical factor that assists adipogenesis. During adipogenesis SREBP-1 stimulates lipogenic gene expression, and peroxisome proliferator-activated receptor γ (PPARγ) enhances perilipin (plin) gene expression, resulting in generating lipid droplets (LDs) to store triacylglycerol (TAG) in adipocytes. Plin coats adipocyte LDs and protects them from lipolysis. Here we show in white adipose tissue (WAT) of plin−/− mice that nuclear active SREBP-1 and its target gene expression, but not nuclear SREBP-2, significantly decreased on attenuated LD formation. When plin−/− mouse embryonic fibroblasts (MEFs) differentiated into adipocytes, attenuated LDs were formed and nuclear SREBP-1 decreased, but enforced plin expression restored them to their original state. Since LDs are largely derived from the endoplasmic reticulum (ER), alterations in the ER cholesterol content were investigated during adipogenesis of 3T3-L1 cells. The ER cholesterol greatly reduced in differentiated adipocytes. The ER cholesterol level in plin−/− WAT was significantly higher than that of wild-type mice, suggesting that increased LD formation caused a change in ER environment along with a decrease in cholesterol. When GFP-SREBP-1 fusion proteins were exogenously expressed in 3T3-L1 cells, a mutant protein lacking the S1P cleavage site was poorly processed during adipogenesis, providing evidence of the increased canonical pathway for SREBP processing in which SREBP-1 is activated by two cleavage enzymes in the Golgi. Therefore, LD biogenesis may create the ER microenvironment favorable for SREBP-1 activation. We describe the novel interplay between LD formation and SREBP-1 activation through a positive feedback loop.

3.1996 Iron-Induced Changes in the Proteome of Trichomonas vaginalis Hydrogenosomes

Beltran, N.C., Horvathova, L., Jedelsky, P.L., Sedinova, M., Rada, P., Marcincikova, M,., Hrdy, I. and Tachezy, J.

*PloS One, **8(5)**, e65148 (2013)*

Iron plays a crucial role in metabolism as a key component of catalytic and redox cofactors, such as heme or iron-sulfur clusters in enzymes and electron-transporting or regulatory proteins. Limitation of iron availability by the host is also one of the mechanisms involved in immunity. Pathogens must regulate their protein expression according to the iron concentration in their environment and optimize their metabolic pathways in cases of limitation through the availability of respective cofactors. *Trichomonas vaginalis*, a sexually transmitted pathogen of humans, requires high iron levels for optimal growth. It is an anaerobe that possesses hydrogenosomes, mitochondrion-related organelles that harbor pathways of energy metabolism and iron-sulfur cluster assembly. We analyzed the proteomes of hydrogenosomes obtained from cells cultivated under iron-rich and iron-deficient conditions employing two-dimensional peptide separation combining IEF and nano-HPLC with quantitative MALDI-MS/MS. We identified 179 proteins, of which 58 were differentially expressed. Iron deficiency led to the upregulation of proteins involved in iron-sulfur cluster assembly and the downregulation of enzymes involved in carbohydrate metabolism. Interestingly, iron affected the expression of only some of multiple protein paralogues, whereas the expression of others was iron independent. This finding indicates a stringent regulation of differentially expressed multiple gene copies in response to changes in the availability of exogenous iron

3.1997 Apolipoprotein AI and High-Density Lipoprotein Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters: ATP-Binding Cassette A-1, ATP-Binding Cassette G-1, and Scavenger Receptor B-1

Umemoto, T., Han, C.Y., Mitra, P., Averill, M.M., Tang, C., Goodspeed, L., Omer, M., Subramanian, S., Wang, S., Hartigh, L.J.D., Wei, H., Kim, E.J., Kim, J., O'Brien, K.D. and Chait, A.

*Cir. Res., **112(10)**, 1345-1354 (2013)*

Rationale: Macrophage accumulation in adipose tissue associates with insulin resistance and increased cardiovascular disease risk. We previously have shown that generation of reactive oxygen species and monocyte chemotactic factors after exposure of adipocytes to saturated fatty acids, such as palmitate, occurs via translocation of NADPH oxidase 4 into lipid rafts (LRs). The anti-inflammatory effects of apolipoprotein AI (apoAI) and high-density lipoprotein (HDL) on macrophages and endothelial cells seem

to occur via cholesterol depletion of LRs. However, little is known concerning anti-inflammatory effects of HDL and apoAI on adipocytes.
Objective: To determine whether apoAI and HDL inhibit inflammation in adipocytes and adipose tissue, and whether this is dependent on LRs.
Methods and Results: In 3T3L-1 adipocytes, apoAI, HDL, and methyl-β-cyclodextrin inhibited chemotactic factor expression. ApoAI and HDL also disrupted LRs, reduced plasma membrane cholesterol content, inhibited NADPH oxidase 4 translocation into LRs, and reduced palmitate-induced reactive oxygen species generation and monocyte chemotactic factor expression. Silencing ATP-binding cassette A-1 abrogated the effect of apoAI, but not HDL, whereas silencing ATP-binding cassette G-1 or scavenger receptor B-1 abrogated the effect of HDL but not apoAI. In vivo, apoAI transgenic mice fed a high-fat, high-sucrose, cholesterol-containing diet showed reduced chemotactic factor and proinflammatory cytokine expression and reduced macrophage accumulation in adipose tissue.
Conclusions: ApoAI and HDL have anti-inflammatory effects in adipocytes and adipose tissue similar to their effects in other cell types. These effects are consistent with disruption and removal of cholesterol from LRs, which are regulated by cholesterol transporters, such as ATP-binding cassette A-1, ATP-binding cassette G-1, and scavenger receptor B-1.

3.1998 Tam41 Is a CDP-Diacylglycerol Synthase Required for Cardiolipin Biosynthesis in Mitochondria
Tamura, Y., Harada, Y., Nishikawa, S-i., Yamano, K., Kamiya, M., Shiota, T., Kuroda, T., Kuge, O., Sesaki, H., Imai, K., Tomil, K. and Endo, T.
*Cell Metabolism, **17(5)**, 709-718 (2013)*

CDP-diacylglycerol (CDP-DAG) is central to the phospholipid biosynthesis pathways in cells. A prevailing view is that only one CDP-DAG synthase named Cds1 is present in both the endoplasmic reticulum (ER) and mitochondrial inner membrane (IM) and mediates generation of CDP-DAG from phosphatidic acid (PA) and CTP. However, we demonstrate here by using yeast *Saccharomyces cerevisiae* as a model organism that Cds1 resides in the ER but not in mitochondria, and that Tam41, a highly conserved mitochondrial maintenance protein, directly catalyzes the formation of CDP-DAG from PA in the mitochondrial IM. We also find that inositol depletion by overexpressing an arrestin-related protein Art5 partially restores the defects of cell growth and CL synthesis in the absence of Tam41. The present findings unveil the missing step of the cardiolipin synthesis pathway in mitochondria as well as the flexibile regulation of phospholipid biosynthesis to respond to compromised CDP-DAG synthesis in mitochondria.

3.1999 Bioanalysis of Eukaryotic Organelles
Satori, C.P., Henderson, M.M., Krautkramer, E.A., Kostal, V., Distefano, M.M. and Arriaga, E.A.
*Chem. Rev., **113(4)**, 2733-2811 (2013)*

No abstract available.

3.2000 Major histocompatibility complex class-II molecules promote targeting of human immunodeficiency virus type 1 virions in late endosomes by enhancing internalization of nascent particles from the plasma membrane
Finzi, A., Periman, M., Bourgeois-Daigneault, M-C., Thibodeau, J. and Cohen, E.A.
*Cell. Microbiol., **15(5)**, 809-822 (2013)*

Productive assembly of human immunodeficiency virus type 1 (HIV-1) takes place, primarily, at the plasma membrane. However, depending on the cell types, a significant proportion of nascent virus particles are internalized and routed to late endosomes. We previously reported that expression of human leucocyte antigen (HLA)-DR promoted a redistribution of Gag in late endosomes and an increased detection of mature virions in these compartments in HeLa and human embryonic kidney 293T model cell lines. Although this redistribution of Gag resulted in a marked decrease of HIV-1 release, the underlying mechanism remained undefined. Here, we provide evidence that expression of HLA-DR at the cell surface induces a redistribution of mature Gag products into late endosomes by enhancing nascent HIV-1 particle internalization from the plasma membrane through a process that relies on the presence of intact HLA-DR α and β-chain cytosolic tails. These findings raise the possibility that major histocompatibility complex class-II molecules might influence endocytic events at the plasma membrane and as a result promote endocytosis of progeny HIV-1 particles.

3.2001 Determination and physiological roles of the glycosylphosphatidylinositol lipid remodelling pathway in yeast

Yoko-o, T., Ichikawa, D., Miyagishi, Y., Kato, A., Umemura, M., Takase, K., Ra, M., Ikeda, K., Taguchi, R. and Jigami, Y.
Mol. Microbiol., ***88(1)***, *140-155 (2013)*

In the yeast *Saccharomyces cerevisiae*, glycosylphosphatidylinositol (GPI)-anchored proteins play important roles in cell wall biogenesis/assembly and the formation of lipid microdomains. The lipid moieties of mature GPI-anchored proteins in yeast typically contain either ceramide moieties or diacylglycerol. Recent studies have identified that the GPI phospholipase A_2 Per1p and *O*-acyltransferase Gup1p play essential roles in diacylglycerol-type lipid remodelling of GPI-anchored proteins, while Cwh43p is involved in the remodelling of lipid moieties to ceramide. It has been generally proposed that phosphatidylinositol with diacylglycerol containing a C26 saturated fatty acid, which is generated by the sequential activity of Per1p and Gup1p, is converted to inositolphosphorylceramide by Cwh43p. In this report, we constructed double-mutant strains defective in lipid remodelling and investigated their growth phenotypes and the lipid moieties of GPI-anchored proteins. Based on our analyses of single- and double-mutants of proteins involved in lipid remodelling, we demonstrate that an alternative pathway, in which lyso-phosphatidylinositol generated by Per1p is used as a substrate for Cwh43p, is involved in the remodelling of GPI lipid moieties to ceramide when the normal sequential pathway is inhibited. In addition, mass spectrometric analysis of lipid species of Flag-tagged Gas1p revealed that Gas1p contains ceramide moieties in its GPI anchor.

3.2002 Motor and sensory neuropathy due to myelin infolding and paranodal damage in a transgenic mouse model of Charcot–Marie–Tooth disease type 1C

Lee, S.M., Sha, D., Mohammed, A.A., Asress, S., Glass, J.D., Chin, L-S. and Li, L.
Human Mol. Genet., ***22(9)***, *1755-1770 (2013)*

Charcot–Marie–Tooth disease type 1C (CMT1C) is a dominantly inherited motor and sensory neuropathy. Despite human genetic evidence linking missense mutations in SIMPLE to CMT1C, the *in vivo* role of CMT1C-linked SIMPLE mutations remains undetermined. To investigate the molecular mechanism underlying CMT1C pathogenesis, we generated transgenic mice expressing either wild-type or CMT1C-linked W116G human SIMPLE. Mice expressing mutant, but not wild type, SIMPLE develop a late-onset motor and sensory neuropathy that recapitulates key clinical features of CMT1C disease. SIMPLE mutant mice exhibit motor and sensory behavioral impairments accompanied by decreased motor and sensory nerve conduction velocity and reduced compound muscle action potential amplitude. This neuropathy phenotype is associated with focally infolded myelin loops that protrude into the axons at paranodal regions and near Schmidt–Lanterman incisures of peripheral nerves. We find that myelin infolding is often linked to constricted axons with signs of impaired axonal transport and to paranodal defects and abnormal organization of the node of Ranvier. Our findings support that SIMPLE mutation disrupts myelin homeostasis and causes peripheral neuropathy via a combination of toxic gain-of-function and dominant-negative mechanisms. The results from this study suggest that myelin infolding and paranodal damage may represent pathogenic precursors preceding demyelination and axonal degeneration in CMT1C patients.

3.2003 Protein Sorting Motifs in the Cytoplasmic Tail of SorCS1 Control Generation of Alzheimer's Amyloid-β Peptide

Lane, R.F., Steele, J.W., Cai, D., Ehrlich, M.E., Attie, A.D. and Gandy, S.
J. Neurosci., ***33(16)***, *7099-7107 (2013)*

Endosomal sorting of the Alzheimer amyloid precursor protein (APP) plays a key role in the biogenesis of the amyloid-β (Aβ) peptide. Genetic lesions underlying Alzheimer's disease (AD) can act by interfering with this physiological process. Specifically, proteins involved in trafficking between endosomal compartments and the trans-Golgi network (TGN) [including the retromer complex (Vps35, Vps26) and its putative receptors (sortilin, SorL1, SorCS1)] have been implicated in the molecular pathology of late-onset AD. Previously, we demonstrated a role for SorCS1 in APP metabolism and Aβ production and, while we implicated a role for the retromer in this regulation, the underlying mechanism remained poorly understood. Here, we provide evidence for a motif within the SorCS1c cytoplasmic tail that, when manipulated, results in perturbed sorting of APP and/or its fragments to endosomal compartments, decreased retrograde TGN trafficking, and increased Aβ production in H4 neuroglioma cells. These perturbations apparently do not involve turnover of the cell surface APP pool, but rather they involve intracellular APP and/or its fragments, downstream of APP endocytosis.

3.2004 **PtdIns(4)P regulates retromer–motor interaction to facilitate dynein–cargo dissociation at the trans-Golgi network**
Niu, Y., Zhang, C., Sun, Z., Hong, Z., Li, K., Sun, D.a, yang, Y., Tian, C., Gong, W. and Liu, J-J.
Nature Cell Biol., ***15(4)***, *417-429 (2013)*

The molecular mechanisms for the retrograde motor dynein–dynactin to unload its cargoes at their final destination remain to be elucidated. In this study, we have investigated the regulatory mechanism underlying release of retromer-associated cargoes at the *trans*-Golgi network (TGN). We report that phosphotidylinositol-4-phosphate (PtdIns(4)P), a Golgi-enriched phosphoinositide, negatively regulates the protein–protein interaction between the $p150^{Glued}$ subunit of dynein–dynactin and the retromer component SNX6. We show that PtdIns(4)P specifically facilitates dissociation of retromer-mediated membranous cargoes from the motor at the TGN and uncover an important function for PtdIns(4)P in the spatial control of retrograde vesicular trafficking to the TGN membrane. PtdIns(4)P also regulates SNX4-mediated retrograde vesicular trafficking to the endocytic recycling compartment by modulating its interaction with dynein. These results establish organelle-specific phosphoinositide regulation of motor–cargo interaction as a mechanism for cargo release by molecular motors at target membrane.

3.2005 **Lc3 Over-Expression Improves Survival and Attenuates Lung Injury Through Increasing Autophagosomal Clearance in Septic Mice**
Lo, S., Yuan, S-S.F., Hsu, C., Cheng, Y.J., Chang, Y-F., Hsueh, H-W., Lee, P-H., and Hsieh, Y-C.
Annals of Surgery, ***257(2)***, *352-363 (2013)*

Objective: To clarify the role of autophagy in sepsis-induced lung injury.
Background: The role of autophagy as a protective or maladaptive response in lung cells during sepsis has not yet been determined. The lack of specificity of the autophagic process has driven the development of new approaches that assessautophagosomes from formation to fusion with lysosomes.
Methods: Sepsis was induced by cecal ligation and puncture (CLP). The autophagic process was manipulated using the pharmacological inhibitors of the autophagy pathway. Green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (LC3) transgenic mice were further used to determine the role of autophagy.
Results: The formation of autophagosomal protein LC3-II progressively accumulated in the lungs over 24 hours after CLP, with the *Lc3* gene expression returning to baseline levels at 24 hours. Autophagosome-lysosome fusion, however, gradually decreased from 8 to 24 hours after CLP, suggesting impaired clearance of autophagosomes rather than upregulation of autophagy in the septic lung. In contrast, transgenic mice overexpressing the *Lc3* gene exhibited increased clearance of autophagosomes and improved survival after CLP. This protective effect was also seen in decreased cell death, inflammatory responses, neutrophil accumulation, albumin leakage, and edema formation. However, blockade of autophagosome-lysosome fusion with bafilomycin A1 abolished the protective effects in transgenic mice. This indicates that *Lc3* transgene attenuates lung injury/inflammation in sepsis, possibly through increasing the clearance of autophagosomes.
Conclusions: Autophagy in the septic lung represents a protective response. However, autophagy, by virtue of excessive autophagosome accumulation, may play a maladaptive role in the late stage of sepsis, leading to acute lung injury.

3.2006 **Negative Regulation of the Novel norpAP24 Suppressor, diehard4, in the Endo-lysosomal Trafficking Underlies Photoreceptor Cell Degeneration**
Lee, J., Song, M. and Hong, S.
PloS Genetics, ***9(6)***, *e1003559 (2013)*

Rhodopsin has been used as a prototype system to investigate G protein-coupled receptor (GPCR) internalization and endocytic sorting mechanisms. Failure of rhodopsin recycling upon light activation results in various degenerative retinal diseases. Accumulation of internalized rhodopsin in late endosomes and the impairment of its lysosomal degradation are associated with unregulated cell death that occurs in dystrophies. However, the molecular basis of rhodopsin accumulation remains elusive. We found that the novel $norpA^{P24}$ suppressor, *diehard4*, is responsible for the inability of endo-lysosomal rhodopsin trafficking and retinal degeneration in *Drosophila* models of retinal dystrophies. We found that *diehard4* encodes *Osiris 21*. Loss of its function suppresses retinal degeneration in $norpA^{P24}$, $rdgC^{306}$, and trp^{1}, but not in $rdgB^{2}$, suggesting a common cause of photoreceptor death. In addition, the loss of *Osiris 21* function shifts the membrane balance between late endosomes and lysosomes as evidenced by smaller late

endosomes and the proliferation of lysosomal compartments, thus facilitating the degradation of endocytosed rhodopsin. Our results demonstrate the existence of negative regulation in vesicular traffic between endosomes and lysosomes. We anticipate that the identification of additional components and an in-depth description of this specific molecular machinery will aid in therapeutic interventions of various retinal dystrophies and GPCR-related human diseases.

3.2007 NHERF2 Protein Mobility Rate Is Determined by a Unique C-terminal Domain That Is Also Necessary for Its Regulation of NHE3 Protein in OK Cells
Yang, J., Singh, V., Cha, B., Chen, T-E., Sarker, R., Murtazina, R., Jin, S., Zachos, N.C., Patterson, G.H., Tse, C.M., Kovbasnjuk, O., Li, X. and Donowitz, M.
J. Biol. Chem., ***288(23)***, *16960-16974 (2013)*

Na^+/H^+ exchanger regulatory factor (NHERF) proteins are a family of PSD-95/Discs-large/ZO-1 (PDZ)-scaffolding proteins, three of which (NHERFs 1-3) are localized to the brush border in kidney and intestinal epithelial cells. All NHERF proteins are involved in anchoring membrane proteins that contain PDZ recognition motifs to form multiprotein signaling complexes. In contrast to their predicted immobility, NHERF1, NHERF2, and NHERF3 were all shown by fluorescence recovery after photobleaching/confocal microscopy to be surprisingly mobile in the microvilli of the renal proximal tubule OK cell line. Their diffusion coefficients, although different among the three, were all of the same magnitude as that of the transmembrane proteins, suggesting they are all anchored in the microvilli but to different extents. NHERF3 moves faster than NHERF1, and NHERF2 moves the slowest. Several chimeras and mutants of NHERF1 and NHERF2 were made to determine which part of NHERF2 confers the slower mobility rate. Surprisingly, the slower mobility rate of NHERF2 was determined by a unique C-terminal domain, which includes a nonconserved region along with the ezrin, radixin, moesin (ERM) binding domain. Also, this C-terminal domain of NHERF2 determined its greater detergent insolubility and was necessary for the formation of larger multiprotein NHERF2 complexes. In addition, this NHERF2 domain was functionally significant in NHE3 regulation, being necessary for stimulation by lysophosphatidic acid of activity and increased mobility of NHE3, as well as necessary for inhibition of NHE3 activity by calcium ionophore 4-Br-A23187. Thus, multiple functions of NHERF2 require involvement of an additional domain in this protein.

3.2008 Intestinal caveolin-1 is important for dietary fatty acid absorption
Siddiqi, S., Sheth, A., patel, F., Barnes, M. and Mansbach II, C.M.
Biochim. Biophys. Acta, ***1831****, 1311-1321 (2013)*

How dietary fatty acids are absorbed into the enterocyte and transported to the ER is not established. We tested the possibility that caveolin-1 containing lipid rafts and endocytic vesicles were involved. Apical brush border membranes took up 15% of albumin bound ^{3}H-oleate whereas brush border membranes from caveolin-1 KO mice took up only 1%. In brush border membranes, the ^{3}H-oleate was in the detergent resistant fraction of an OptiPrep gradient. On OptiPrep gradients of intestinal cytosol, we also found the ^{3}H-oleate in the detergent resistant fraction, separate from OptiPrep gradients spiked with ^{3}H-oleate or ^{3}H-triacylglycerol. Caveolin-1 immuno-depletion of cytosol removed 91% of absorbed ^{3}H-oleate whereas immuno-depletion using IgG, or anti-caveolin-2 or -3 or anti-clathrin antibodies removed 20%. Electron microscopy showed the presence of caveolin-1 containing vesicles in WT mouse cytosol that were 4 fold increased by feeding intestinal sacs 1 mM oleate. No vesicles were seen in caveolin-1 KO mouse cytosol. Caveolin-1 KO mice gained less weight on a 23% fat diet and had increased fat in their stool compared to WT mice. We conclude that dietary fatty acids are absorbed by caveolae in enterocyte brush border membranes, are endocytosed, and transported in cytosol in caveolin-1 containing endocytic vesicles.

3.2009 Apical sorting of lysoGPI-anchored proteins occurs independent of association with detergent-resistant membranes but dependent on their N-glycosylation
Castillon, G.A., Michon, L. and Watanabe, R.
Mol. Biol. Cell, ***24****, 2021-2033 (2013)*

Most glycosylphosphatidylinositol-anchored proteins (GPI-APs) are located at the apical surface of epithelial cells. The apical delivery of GPI-APs is believed to result from their association with lipid rafts. We find that overexpression of C-terminally tagged PGAP3 caused predominant production of lysoGPI-APs, an intermediate precursor in the GPI lipid remodeling process in Madin–Darby canine kidney cells. In these cells, produced lysoGPI-APs are not incorporated into detergent-resistant membranes (DRMs) but still are delivered apically, suggesting that GPI-AP association with DRMs is not necessary for apical

targeting. In contrast, apical transport of both fully remodeled and lyso forms of GPI-APs is dependent on N-glycosylation, confirming a general role of N-glycans in apical protein transport. We also find that depletion of cholesterol causes apical-to-basolateral retargeting not only of fully remodeled GPI-APs, but also of lysoGPI-APs, as well as endogenous soluble and transmembrane proteins that would normally be targeted to the apical membrane. These findings confirm the essential role for cholesterol in the apical protein targeting and further demonstrate that the mechanism of cholesterol-dependent apical sorting is not related to DRM association of GPI-APs.

3.2010 Stress-Induced Outer Membrane Vesicle Production by *Pseudomonas aeruginosa*
MacDonald, I.A. and Kuehn, M.J.
J. Bacteriol., ***195(13)***, *2971-2981 (2013)*

As an opportunistic Gram-negative pathogen, Pseudomonas aeruginosa must be able to adapt and survive changes and stressors in its environment during the course of infection. To aid survival in the hostile host environment, P. aeruginosa has evolved defense mechanisms, including the production of an exopolysaccharide capsule and the secretion of a myriad of degradative proteases and lipases. The production of outer membrane-derived vesicles (OMVs) serves as a secretion mechanism for virulence factors as well as a general bacterial response to envelope-acting stressors. This study investigated the effect of sublethal physiological stressors on OMV production by P. aeruginosa and whether the Pseudomonas quinolone signal (PQS) and the MucD periplasmic protease are critical mechanistic factors in this response. Exposure to some environmental stressors was determined to increase the level of OMV production as well as the activity of AlgU, the sigma factor that controls MucD expression. Overexpression of AlgU was shown to be sufficient to induce OMV production; however, stress-induced OMV production was not dependent on activation of AlgU, since stress caused increased vesiculation in strains lacking *algU*. We further determined that MucD levels were not an indicator of OMV production under acute stress, and PQS was not required for OMV production under stress or unstressed conditions. Finally, an investigation of the response of P. aeruginosa to oxidative stress revealed that peroxide-induced OMV production requires the presence of B-band but not A-band lipopolysaccharide. Together, these results demonstrate that distinct mechanisms exist for stress-induced OMV production in P. aeruginosa.

3.2011 Lipidome analysis of rotavirus-infected cells confirms the close interaction of lipid droplets with viroplasms
Gaunt, E.R., Zhang, Q., Cheung, W., Wakelam, M.J.O., Lever, A.M. and Desselberger, U.
J. Gen. Virol., ***94***, *1576-1586 (2013)*

Rotaviruses (RVs) cause acute gastroenteritis in infants and young children, and are globally distributed. Within the infected host cell, RVs establish replication complexes in viroplasms (‘viral factories’) to which lipid droplet organelles are recruited. To further understand this recently discovered phenomenon, the lipidomes of RV-infected and uninfected MA104 cells were investigated. Cell lysates were subjected to equilibrium ultracentrifugation through iodixanol gradients. Fourteen different classes of lipids were differentiated by mass spectrometry. The concentrations of virtually all lipids were elevated in RV-infected cells. Fractions of low density (1.11–1.15 g ml^{-1}), in which peaks of the RV dsRNA genome and lipid droplet- and viroplasm-associated proteins were observed, contained increased amounts of lipids typically found concentrated in the cellular organelle lipid droplets, confirming the close interaction of lipid droplets with viroplasms. A decrease in the ratio of the amounts of surface to internal components of lipid droplets upon RV infection suggested that the lipid droplet–viroplasm complexes became enlarged.

3.2012 Calpain-Mediated Processing of Adenylate Cyclase Toxin Generates a Cytosolic Soluble Catalytically Active N-Terminal Domain
Uribe, K.B., Etxebarria, A., Martin, C. and Ostolaza, H.
PloS One, ***8(6)***, *e67648 (2013)*

Bordetella pertussis, the whooping cough pathogen, secretes several virulence factors among which adenylate cyclase toxin (ACT) is essential for establishment of the disease in the respiratory tract. ACT weakens host defenses by suppressing important bactericidal activities of the phagocytic cells. Up to now, it was believed that cell intoxication by ACT was a consequence of the accumulation of abnormally high levels of cAMP, generated exclusively beneath the host plasma membrane by the toxin N-terminal catalytic adenylate cyclase (AC) domain, upon its direct translocation across the lipid bilayer. Here we show that host calpain, a calcium-dependent Cys-protease, is activated into the phagocytes by a toxin-triggered calcium rise, resulting in the proteolytic cleavage of the toxin N-terminal domain that releases a

catalytically active “soluble AC”. The calpain-mediated ACT processing allows trafficking of the “soluble AC” domain into subcellular organella. At least two strategic advantages arise from this singular toxin cleavage, enhancing the specificity of action, and simultaneously preventing an indiscriminate activation of cAMP effectors throughout the cell. The present study provides novel insights into the toxin mechanism of action, as the calpain-mediated toxin processing would confer ACT the capacity for a space- and time-coordinated production of different cAMP “pools”, which would play different roles in the cell pathophysiology.

3.2013 Proteomic Identification of VEGF-dependent Protein Enrichment to Membrane Caveolar-raft Microdomains in Endothelial Progenitor Cells
Chilla, A., Magherini, F., Margheri, F., Laurenzanat, A., Gamberit, T., Bini, L., Bianchi, L., Danza, G., Mazzanti, B., Serrati, S., Modesti, A., Del Rosso, M. and Fibbi, G.
Mol. Cell. Proteomics, ***12***, *1926-1938 (2013)*

Endothelial cell caveolar-rafts are considered functional platforms that recruit several pro-angiogenic molecules to realize an efficient angiogenic program. Here we studied the differential caveolar-raft protein composition of endothelial colony-forming cells following stimulation with VEGF, which localizes in caveolae on interaction with its type-2 receptor. Endothelial colony-forming cells are a cell population identified in human umbilical blood that show all the properties of an endothelial progenitor cell and a high proliferative rate. Two-dimensional gel electrophoresis analysis was coupled with mass spectrometry to identify candidate proteins. The twenty-eight differentially expressed protein spots were grouped according to their function using Gene Ontology classification. In particular, functional categories relative to cell death inhibition and hydrogen peroxide metabolic processes resulted enriched. In these categories, Peroxiredoxin-2 and 6, that control hydrogen peroxide metabolic processes, are the main enriched molecules together with the anti-apoptotic 78 kDa glucose regulated protein. Some of the proteins we identified had never before identified as caveolar-raft components. Other identified proteins include calpain small subunit-1, known to mediates angiogenic response to VEGF, gelsolin, which regulates stress fiber assembly, and annexin A3, an angiogenic mediator that induces VEGF production. We validated the functional activity of the above proteins, showing that the siRNA silencing of these resulted in the inhibition of capillary morphogenesis. Overall, our data show that VEGF stimulation triggers the caveolar-raft recruitment of proteins that warrant a physiological amount of reactive oxygen species to maintain a proper angiogenic function of endothelial colony-forming cells and preserve the integrity of the actin cytoskeleton.

3.2014 Impaired Very Long-chain Acyl-CoA β-Oxidation in Human X-linked Adrenoleukodystrophy Fibroblasts Is a Direct Consequence of ABCD1 Transporter Dysfunction
Wiesinger, C., Kunze, M., Regelsberger, G., Forss-Petter, S. and Berger, J.
J. Biol. Chem., ***288(26)***, *19269-19279 (2013)*

X-linked adrenoleukodystrophy (X-ALD), an inherited peroxisomal disorder, is caused by mutations in the *ABCD1* gene encoding the peroxisomal ATP-binding cassette (ABC) transporter ABCD1 (adrenoleukodystrophy protein, ALDP). Biochemically, X-ALD is characterized by an accumulation of very long-chain fatty acids and partially impaired peroxisomal β-oxidation. In this study, we used primary human fibroblasts from X-ALD and Zellweger syndrome patients to investigate the peroxisomal β-oxidation defect. Our results show that the degradation of C26:0-CoA esters is as severely impaired as degradation of unesterified very long-chain fatty acids in X-ALD and is abolished in Zellweger syndrome. Interestingly, the β-oxidation rates for both C26:0-CoA and C22:0-CoA were similarly affected, although C22:0 does not accumulate in patient fibroblasts. Furthermore, we show that the β-oxidation defect in X-ALD is directly caused by ABCD1 dysfunction as blocking ABCD1 function with a specific antibody reduced β-oxidation to levels observed in X-ALD fibroblasts. By quantification of mRNA and protein levels of the peroxisomal ABC transporters and by blocking with specific antibodies, we found that residual β-oxidation activity toward C26:0-CoA in X-ALD fibroblasts is mediated by ABCD3, although the efficacy of ABCD3 appeared to be much lower than that of ABCD1. Finally, using isolated peroxisomes, we show that β-oxidation of C26:0-CoA is independent of additional CoA but requires a cytosolic factor of >10-kDa molecular mass that is resistant to *N*-ethylmaleimide and heat inactivation. In conclusion, our findings in human cells suggest that, in contrast to yeast cells, very long-chain acyl-CoA esters are transported into peroxisomes by ABCD1 independently of additional synthetase activity.

3.2015 Exosomes Derived from HIV-1-infected Cells Contain Trans-activation Response Element RNA
Narayanan, A., Iordanskiy, S., Das, r., Duyne, R., Santos, S., Jaworski, E., Guendel, I., Sampey, G., Dalby, E., Iglesiaas-Ussel, M., Popratiloff, A., Hakami, R., Kehn-Hall, K., Young, M., Subra, C., Gilbert, C., Bailey, C., Romerio, F. and Kashanchi, F.
J. Biol. Chem., ***288(27)***, *20014-20033 (2013)*

Exosomes are nano-sized vesicles produced by healthy and virus-infected cells. Exosomes derived from infected cells have been shown to contain viral microRNAs (miRNAs). HIV-1 encodes its own miRNAs that regulate viral and host gene expression. The most abundant HIV-1-derived miRNA, first reported by us and later by others using deep sequencing, is the trans-activation response element (TAR) miRNA. In this study, we demonstrate the presence of TAR RNA in exosomes from cell culture supernatants of HIV-1-infected cells and patient sera. TAR miRNA was not in Ago2 complexes outside the exosomes but enclosed within the exosomes. We detected the host miRNA machinery proteins Dicer and Drosha in exosomes from infected cells. We report that transport of TAR RNA from the nucleus into exosomes is a CRM1 (chromosome region maintenance 1)-dependent active process. Prior exposure of naive cells to exosomes from infected cells increased susceptibility of the recipient cells to HIV-1 infection. Exosomal TAR RNA down-regulated apoptosis by lowering Bim and Cdk9 proteins in recipient cells. We found 10^4–10^6 copies/ml TAR RNA in exosomes derived from infected culture supernatants and 10^3 copies/ml TAR RNA in the serum exosomes of highly active antiretroviral therapy-treated patients or long term nonprogressors. Taken together, our experiments demonstrated that HIV-1-infected cells produced exosomes that are uniquely characterized by their proteomic and RNA profiles that may contribute to disease pathology in AIDS.

3.2016 The effect of organelle discovery upon sub-cellular protein localisation
Breckels, L.M:, Gatto, L., Christoforou, A., Groen, A.J., Lilley, K.S. and Trotter, M.W.B.
J. Proteomics, ***88***, *129-140 (2013)*

Prediction of protein sub-cellular localisation by employing quantitative mass spectrometry experiments is an expanding field. Several methods have led to the assignment of proteins to specific subcellular localisations by partial separation of organelles across a fractionation scheme coupled with computational analysis.
Methods developed to analyse organelle data have largely employed supervised machine learning algorithms to map unannotated abundance profiles to known protein-organelle associations. Such approaches are likely to make association errors if organelle-related groupings present in experimental output are not included in data used to create a protein-organelle classifier. Currently, there is no automated way to detect organelle-specific clusters within such datasets.
In order to address the above issues we adapted a phenotype discovery algorithm, originally created to filter image-based output for RNAi screens, to identify putative subcellular groupings in organelle proteomics experiments. We were able to mine datasets to a deeper level and extract interesting phenotype clusters for more comprehensive evaluation in an unbiased fashion upon application of this approach. Organelle-related protein clusters were identified beyond those sufficiently annotated for use as training data. Furthermore, we propose avenues for the incorporation of observations made into general practice for the classification of protein-organelle membership from quantitative MS experiments.
Biological significance
Protein sub-cellular localisation plays an important role in molecular interactions, signalling and transport mechanisms. The prediction of protein localisation by quantitative mass-spectrometry (MS) proteomics is a growing field and an important endeavour in improving protein annotation. Several such approaches use gradient-based separation of cellular organelle content to measure relative protein abundance across distinct gradient fractions. The distribution profiles are commonly mapped in silico to known protein-organelle associations via supervised machine learning algorithms, to create classifiers that associate unannotated proteins to specific organelles. These strategies are prone to error, however, if organelle-related groupings present in experimental output are not represented, for example owing to the lack of existing annotation, when creating the protein-organelle mapping. Here, the application of a phenotype discovery approach to LOPIT gradient-based MS data identifies candidate organelle phenotypes for further evaluation in an unbiased fashion. Software implementation and usage guidelines are provided for application to wider protein-organelle association experiments. In the wider context, semi-supervised organelle discovery is discussed as a paradigm with which to generate new protein annotations from MS-based organelle proteomics experiments. This article is part of a Special Issue entitled: New Horizons and Applications for Proteomics [EuPA 2012].

3.2017 **Stx5 is a novel interactor of VLDL-R to affect its intracellular trafficking and processing**
Wagner, T., Dieckmann, M., Jaeger, S., Weggen, S. and Pietrzik, C.U.
*Exp. Cell Res., **319**, 1956-1972 (2013)*

We identified syntaxin 5 (Stx5), a protein involved in intracellular vesicle trafficking, as a novel interaction partner of the very low density lipoprotein (VLDL)-receptor (VLDL-R), a member of the LDL-receptor family. In addition, we investigated the effect of Stx5 on VLDL-R maturation, trafficking and processing. Here, we demonstrated mutual association of both proteins using several *in vitro* approaches. Furthermore, we detected a special maturation phenotype of VLDL-R resulting from Stx5 overexpression. We found that Stx5 prevented advanced Golgi-maturation of VLDL-R, but did not cause accumulation of the immature protein in ER, ER to Golgi compartments, or *cis*-Golgi ribbon, the main expression sites of Stx5. Rather more, abundantly present Stx5 was capable of translocating ER-/N-glycosylated VLDL-R to the plasma membrane, and thus was insensitive to BFA treatment and low temperature. Furthermore, abundant presence of Stx5 significantly interfered with VLDL-R reaching the *trans*-Golgi network. Based on our findings, we postulate that Stx5 can directly bind to the *C*-terminal domain of VLDL-R, thereby influencing the receptor′ s glycosylation, trafficking and processing characteristics. Resulting from that, we further suggest that Stx5 might play a role in modulating VLDL-R physiology by participating in an abrasively described or completely novel Golgi-bypass pathway.

3.2018 **EphrinA2 Regulates Clathrin Mediated KSHV Endocytosis in Fibroblast Cells by Coordinating Integrin-Associated Signaling and c-Cbl Directed Polyubiquitination**
Dutta, D., Chakraborty, S., Bandyopadhyay, C., Veettil, M.V., Ansari, M.A., Singh, V.V. and Chandran, B.
*PloS One, **9(7)**, e1003510 (2013)*

Kaposi's sarcoma-associated herpesvirus (KSHV) interacts with human dermal endothelial cell surface tyrosine kinase EphrinA2 (EphA2) and integrins (α3β1 and αVβ3) in the lipid raft (LR) region, and EphA2 regulates macropinocytic virus entry by coordinating integrin-c-Cbl associated signaling. In contrast, KSHV enters human foreskin fibroblast (HFF) cells by LR-independent clathrin mediated endocytosis. The present studies conducted to identify the key molecules regulating KSHV entry in HFF cells showed that KSHV induces association with integrins (αVβ5, αVβ3 and α3β1) and EphA2 in non-LR regions early during infection and activates EphA2, which in turn associates with phosphorylated c-Cbl, myosin IIA, FAK, Src, and PI3-K, as well as clathrin and its adaptor AP2 and effector Epsin-15 proteins. EphA2 knockdown significantly reduced these signal inductions, virus internalization and gene expression. c-Cbl knockdown ablated the c-Cbl mediated K63 type polyubiquitination of EphA2 and clathrin association with EphA2 and KSHV. Mutations in EphA2's tyrosine kinase domain (TKD) or sterile alpha motif (SAM) abolished its interaction with c-Cbl. Mutations in tyrosine kinase binding (TKB) or RING finger (RF) domains of c-Cbl resulted in very poor association of c-Cbl with EphA2 and decreased EphA2 polyubiquitination. These studies demonstrated the contributions of these domains in EphA2 and c-Cbl association, EphA2 polyubiquitination and virus-EphA2 internalization. Collectively, these results revealed for the first time that EphA2 influences the tyrosine phosphorylation of clathrin, the role of EphA2 in clathrin mediated endocytosis of a virus, and c-Cbl mediated EphA2 polyubiquitination directing KSHV entry in HFF cells via coordinated signal induction and progression of endocytic events, all of which suggest that targeting EphA2 and c-Cbl could block KSHV entry and infection.

3.2019 **Oncogenic H-Ras Reprograms Madin-Darby Canine Kidney (MDCK) Cell-derived Exosomal Proteins Following Epithelial-Mesenchymal Transition**
Tauro, B.J., Mathias, R.A., Greening, D.W., Gopal, S.K., Ji, H., Kapp, E.A., Coleman, B.M., Hill, A.F., Kusebauch, U., Hallows, J.L., Shteynberg, D., Moritz, R.L., Zhu, H-J. and Simpson, R.J.
*Mol. Cell. Proteomics, **12(8)**, 2148-2159 (2013)*

Epithelial-mesenchymal transition (EMT) is a highly conserved morphogenic process defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. EMT is associated with increased aggressiveness, invasiveness, and metastatic potential in carcinoma cells. To assess the contribution of extracellular vesicles following EMT, we conducted a proteomic analysis of exosomes released from Madin-Darby canine kidney (MDCK) cells, and MDCK cells transformed with oncogenic H-Ras (21D1 cells). Exosomes are 40–100 nm membranous vesicles originating from the inward budding of late endosomes and multivesicular bodies and are released from cells on fusion of multivesicular bodies with the plasma membrane. Exosomes from MDCK cells (MDCK-Exos) and 21D1 cells (21D1-Exos) were purified from cell culture media using density gradient centrifugation (OptiPrep™), and protein

content identified by GeLC-MS/MS proteomic profiling. Both MDCK- and 21D1-Exos populations were morphologically similar by cryo-electron microscopy and contained stereotypical exosome marker proteins such as TSG101, Alix, and CD63. In this study we show that the expression levels of typical EMT hallmark proteins seen in whole cells correlate with those observed in MDCK- and 21D1-Exos, *i.e.* reduction of characteristic inhibitor of angiogenesis, thrombospondin-1, and epithelial markers E-cadherin, and EpCAM, with a concomitant up-regulation of mesenchymal makers such as vimentin. Further, we reveal that 21D1-Exos are enriched with several proteases (*e.g.* MMP-1, -14, -19, ADAM-10, and ADAMTS1), and integrins (*e.g.* ITGB1, ITGA3, and ITGA6) that have been recently implicated in regulating the tumor microenvironment to promote metastatic progression. A salient finding of this study was the unique presence of key transcriptional regulators (*e.g.* the master transcriptional regulator YBX1) and core splicing complex components (*e.g.* SF3B1, SF3B3, and SFRS1) in mesenchymal 21D1-Exos. Taken together, our findings reveal that exosomes from Ras-transformed MDCK cells are reprogrammed with factors which may be capable of inducing EMT in recipient cells.

3.2020 **Sorting of GLUT4 into its insulin-sensitive store requires the Sec1/Munc18 protein mVps45**
Roccisana, J.-, Sadler, J.B.A., Bryant, N.J. and Gould, G.W.
*Mol. Biol. Cell, **24**, 2389-2397 (2013)*

Insulin stimulates glucose transport in fat and muscle cells by regulating delivery of the facilitative glucose transporter, glucose transporter isoform 4 (GLUT4), to the plasma membrane. In the absence of insulin, GLUT4 is sequestered away from the general recycling endosomal pathway into specialized vesicles, referred to as GLUT4-storage vesicles. Understanding the sorting of GLUT4 into this store is a major challenge. Here we examine the role of the Sec1/Munc18 protein mVps45 in GLUT4 trafficking. We show that mVps45 is up-regulated upon differentiation of 3T3-L1 fibroblasts into adipocytes and is expressed at stoichiometric levels with its cognate target–soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor, syntaxin 16. Depletion of mVps45 in 3T3-L1 adipocytes results in decreased GLUT4 levels and impaired insulin-stimulated glucose transport. Using subcellular fractionation and an in vitro assay for GLUT4-storage vesicle formation, we show that mVps45 is required to correctly traffic GLUT4 into this compartment. Collectively our data reveal a crucial role for mVps45 in the delivery of GLUT4 into its specialized, insulin-regulated compartment.

3.2021 **An Inhibitor of the δPKC Interaction with the d Subunit of F1Fo ATP Synthase Reduces Cardiac Troponin I Release from Ischemic Rat Hearts: Utility of a Novel Ammonium Sulfate Precipitation Technique**
Ogbi, M., Obi, I. and Johnson, J.A.
*PloS One, **8(8)**, e70580 (2013)*

We have previously reported protection against hypoxic injury by a cell-permeable, mitochondrially-targeted δPKC-d subunit of F_1Fo ATPase (dF_1Fo) interaction inhibitor [NH_2-YGRKKRRQRRRMLA TRALSLIGKRAISTSVCAGRKLALKTIDWVSFDYKDDDDK-COOH]in neonatal cardiac myo-cytes. In the present work we demonstrate the partitioning of this peptide to the inner membrane and matrix of mitochondria when it is perfused into isolated rat hearts. We also used ammonium sulfate ($(NH_4)_2SO_4$) and chloroform/methanol precipitation of heart effluents to demonstrate reduced card-iac troponin I (cTnI) release from ischemic rat hearts perfused with this inhibitor. 50% $(NH_4)_2SO_4$ saturation of perfusates collected from Langendorff rat heart preparations optimally precipitated cTnI, allowing its detection in Western blots. In hearts receiving 20 min of ischemia followed by 30, or 60 min of reperfusion, the Mean±S.E. (n = 5) percentage of maximal cTnI release was 30±7 and 60±17, respectively, with additional cTnI release occurring after 150 min of reperfusion. Perfusion of hearts with the δPKC-dF_1Fo interaction inhibitor, prior to 20 min of ischemia and 60–150 min of reperfusion, reduced cTnI release by 80%. Additionally, we found that when soybean trypsin inhibitor (SBTI), was added to rat heart effluents, it could also be precipitated using $(NH_4)_2SO_4$ and detected in western blots. This provided a convenient method for normalizing protein recoveries between groups. Our results support the further development of the δPKC-dF_1Fo inhibitor as a potential therapeutic for combating cardiac ischemic injury. In addition, we have developed an improved method for the detection of cTnI release from perfused rat hearts.

3.2022 **Wip1 suppresses apoptotic cell death through direct dephosphorylation of BAX in response to γ-radiation**
Song, J-Y., Ryu, S-H., Cho, Y.M., Kim, Y.S., Lee, B-M., Lee, S-W. and Choi, J.
*Cell Death and Disease, **4**, e744 (2013)*

Wild-type p53-induced phosphatase 1 (Wip1) is a p53-inducible serine/threonine phosphatase that switches off DNA damage checkpoint responses by the dephosphorylation of certain proteins (i.e. p38 mitogen-activated protein kinase, p53, checkpoint kinase 1, checkpoint kinase 2, and uracil DNA glycosylase) involved in DNA repair and the cell cycle checkpoint. Emerging data indicate that Wip1 is amplified or overexpressed in various human tumors, and its detection implies a poor prognosis. In this study, we show that Wip1 interacts with and dephosphorylates BAX to suppress BAX-mediated apoptosis in response to γ-irradiation in prostate cancer cells. Radiation-resistant LNCaP cells showed dramatic increases in Wip1 levels and impaired BAX movement to the mitochondria after γ-irradiation, and these effects were reverted by a Wip1 inhibitor. These results show that Wip1 directly interacts with and dephosphorylates BAX. Dephosphorylation occurs at threonines 172, 174 and 186, and BAX proteins with mutations at these sites fail to translocate efficiently to the mitochondria following cellular γ-irradiation. Overexpression of Wip1 and BAX, but not phosphatase-dead Wip1, in BAX-deficient cells strongly reduces apoptosis. Our results suggest that BAX dephosphorylation of Wip1 phosphatase is an important regulator of resistance to anticancer therapy. This study is the first to report the downregulation of BAX activity by a protein phosphatase.

3.2023 The ER–Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis
Ge, L., Melville, D., Zhang, M. and Schekman, R.
eLife, ***2***, *e00947 (2013)*

Autophagy is a catabolic process for bulk degradation of cytosolic materials mediated by double-membraned autophagosomes. The membrane determinant to initiate the formation of autophagosomes remains elusive. Here, we establish a cell-free assay based on LC3 lipidation to define the organelle membrane supporting early autophagosome formation. In vitro LC3 lipidation requires energy and is subject to regulation by the pathways modulating autophagy in vivo. We developed a systematic membrane isolation scheme to identify the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) as a primary membrane source both necessary and sufficient to trigger LC3 lipidation in vitro. Functional studies demonstrate that the ERGIC is required for autophagosome biogenesis in vivo. Moreover, we find that the ERGIC acts by recruiting the early autophagosome marker ATG14, a critical step for the generation of preautophagosomal membranes.

3.2024 Interaction Domains of Sos1/Grb2 Are Finely Tuned for Cooperative Control of Embryonic Stem Cell Fate
Findlay, G.M. et al
Cell, ***152(5)***, *1008-1020 (2013)*

Metazoan evolution involves increasing protein domain complexity, but how this relates to control of biological decisions remains uncertain. The Ras guanine nucleotide exchange factor (RasGEF) Sos1 and its adaptor Grb2 are multidomain proteins that couple fibroblast growth factor (FGF) signaling to activation of the Ras-Erk pathway during mammalian development and drive embryonic stem cells toward the primitive endoderm (PrE) lineage. We show that the ability of Sos1/Grb2 to appropriately regulate pluripotency and differentiation factors and to initiate PrE development requires collective binding of multiple Sos1/Grb2 domains to their protein and phospholipid ligands. This provides a cooperative system that only allows lineage commitment when all ligand-binding domains are occupied. Furthermore, our results indicate that the interaction domains of Sos1 and Grb2 have evolved so as to bind ligands not with maximal strength but with specificities and affinities that maintain cooperativity. This optimized system ensures that PrE lineage commitment occurs in a timely and selective manner during embryogenesis.

3.2025 Cell-Cell Communication between Malaria-Infected Red Blood Cells via Exosome-like Vesicles
Regev-Rudzki, N., Wilson, D.W., carvalho, T., Sisquella, X., Coleman, B.M., Rug, M., Bursac, D., Angrisano, F., Gee, M., Hill., A.F. and Baum, J.
Cell, ***153(5)***, *1120-1133 (2013)*

Cell-cell communication is an important mechanism for information exchange promoting cell survival for the control of features such as population density and differentiation. We determined that *Plasmodium falciparum*-infected red blood cells directly communicate between parasites within a population using exosome-like vesicles that are capable of delivering genes. Importantly, communication via exosome-like vesicles promotes differentiation to sexual forms at a rate that suggests that signaling is involved. Furthermore, we have identified a *P. falciparum* protein, PfPTP2, that plays a key role in efficient

communication. This study reveals a previously unidentified pathway of *P. falciparum* biology critical for survival in the host and transmission to mosquitoes. This identifies a pathway for the development of agents to block parasite transmission from the human host to the mosquito.

3.2026 Large protein complexes retained in the ER are dislocated by non-COPII vesicles and degraded by selective autophagy

Le Fourn, V., Park, S., Jang, I., Gaplovskaa-Kysela, K., Guhl, B., Lee, Y., Cho, J.W:, Zuber, C. and Roth, J.

Cell Mol. Life Sci., ***70(11),*** *1985-2002 (2013)*

Multisubunit protein complexes are assembled in the endoplasmic reticulum (ER). Existing pools of single subunits and assembly intermediates ensure the efficient and rapid formation of complete complexes. While being kinetically beneficial, surplus components must be eliminated to prevent potentially harmful accumulation in the ER. Surplus single chains are cleared by the ubiquitin–proteasome system. However, the fate of not secreted assembly intermediates of multisubunit proteins remains elusive. Here we show by high-resolution double-label confocal immunofluorescence and immunogold electron microscopy that naturally occurring surplus fibrinogen Aα–γ assembly intermediates in HepG2 cells are dislocated together with EDEM1 from the ER to the cytoplasm in ER-derived vesicles not corresponding to COPII-coated vesicles originating from the transitional ER. This route corresponds to the novel ER exit path we have previously identified for EDEM1 (Zuber et al. Proc Natl Acad Sci USA 104:4407–4412, 2007). In the cytoplasm, detergent-insoluble aggregates of fibrinogen Aα–γ dimers develop that are targeted by the selective autophagy cargo receptors p62/SQSTM1 and NBR1. These aggregates are degraded by selective autophagy as directly demonstrated by high-resolution microscopy as well as biochemical analysis and inhibition of autophagy by siRNA and kinase inhibitors. Our findings demonstrate that different pathways exist in parallel for ER-to-cytoplasm dislocation and subsequent proteolytic degradation of large luminal protein complexes and of surplus luminal single-chain proteins. This implies that ER-associated protein degradation (ERAD) has a broader function in ER proteostasis and is not limited to the elimination of misfolded glycoproteins.

3.2027 Palmitoylation of Amyloid Precursor Protein Regulates Amyloidogenic Processing in Lipid Rafts

Bhattacharyya, R., Barren, C. and Kovacs, D.M.

J. Neurosci., ***33(27)****, 11169- 11183 (2013)*

Brains of patients affected by Alzheimer's disease (AD) contain large deposits of aggregated amyloid β-protein (Aβ). Only a small fraction of the amyloid precursor protein (APP) gives rise to Aβ. Here, we report that ~10% of APP undergoes a post-translational lipid modification called palmitoylation. We identified the palmitoylation sites in APP at Cys^{186} and Cys^{187}. Surprisingly, point mutations introduced into these cysteines caused nearly complete ER retention of APP. Thus, either APP palmitoylation or disulfide bridges involving these Cys residues appear to be required for ER exit of APP. In later compartments, palmitoylated APP (*pal*APP) was specifically enriched in lipid rafts. *In vitro* BACE1 cleavage assays using cell or mouse brain lipid rafts showed that APP palmitoylation enhanced BACE1-mediated processing of APP. Interestingly, we detected an age-dependent increase in endogenous mouse brain *pal*APP levels. Overexpression of selected DHHC palmitoyl acyltransferases increased palmitoylation of APP and doubled Aβ production, while two palmitoylation inhibitors reduced *pal*APP levels and APP processing. We have found previously that acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibition led to impaired APP processing. Here we demonstrate that pharmacological inhibition or genetic inactivation of ACAT decrease lipid raft *pal*APP levels by up to 76%, likely resulting in impaired APP processing. Together, our results indicate that APP palmitoylation enhances amyloidogenic processing by targeting APP to lipid rafts and enhancing its BACE1-mediated cleavage. Thus, inhibition of *pal*APP formation by ACAT or specific palmitoylation inhibitors would appear to be a valid strategy for prevention and/or treatment of AD.

3.2028 Mammalian SOD2 is exclusively located in mitochondria and not present in peroxisomes

Karnati, S., Lüers, G. and Pfreimer, S.

Histochem. Cell. Biol., ***140(2)****, 105-117 (2013)*

Superoxide dismutases (SODs) are metalloenzymes that belong to the essential antioxidant enzyme systems of virtually all oxygen-respiring organisms. SODs catalyze the dismutation of highly reactive superoxide radicals into hydrogen peroxide and molecular oxygen. For the subcellular localization of the manganese superoxide dismutase (SOD2) in eukaryotic cells, a dual mitochondrial localization and

peroxisomal localization were proposed in the literature. However, our own observation from immunofluorescence preparations of human and mouse tissues suggested that SOD2 serves as an excellent marker protein for mitochondria but never co-localized with peroxisomes. To clarify whether our observations were correct, we have carefully reinvestigated the subcellular localization of SOD2 using sensitive double-immunofluorescence methods on frozen and paraffin sections as well as in cell culture preparations. In addition, ultrastructural analyses were performed with post-embedding immunoelectron microscopy on LR White sections as well as labeling of ultrathin cryosections with various immunogold techniques. In all morphological experiments, the SOD2 localization was compared to one of the catalase, a typical marker protein for peroxisomes, solely localized in these organelles. Moreover, biochemical subcellular fractions of mouse liver was used to isolate enriched organelles and highly purified peroxisomal fractions for Western blot analyses of the exact subcellular distributions of SOD2 and catalase. All results with the various methodologies, tissues, and cell types used revealed that catalase and SOD2 were always confined to distinct and separate subcellular compartments. SOD2 was unequivocally in mitochondria, but never present in peroxisomes. Furthermore, our results are supported by accumulating database information on organelle proteomes that also indicate that SOD2 is a pure mitochondrial protein.

3.2029 Membrane cholesterol modulates the hyaluronan-binding ability of CD44 in T lymphocytes and controls rolling under shear flow
Murai, T., Sato, C., Sato, M., Nishiyama, H., Suga, M., Mio, K. and Kawashima, H.
J. Cell Sci., ***126(15)****, 3284-3294 (2013)*

The adhesion of circulating lymphocytes to the surface of vascular endothelial cells is important for their recruitment from blood to secondary lymphoid organs and to inflammatory sites. CD44 is a key adhesion molecule for this interaction and its ligand-binding ability is tightly regulated. Here we show that the hyaluronan-binding ability of CD44 in T cells is upregulated by the depletion of membrane cholesterol with methyl-β-cyclodextrin (MβCD), which disintegrates lipid rafts, i.e. cholesterol- and sphingolipid-enriched membrane microdomains. Increasing concentrations of MβCD led to a dose-dependent decrease in cellular cholesterol content and to upregulation of hyaluronan binding. Additionally, a cholesterol-binding agent filipin also increased hyaluronan binding. Cholesterol depletion caused CD44 to be dispersed from cholesterol-enriched membrane microdomains. Cholesterol depletion also increased the number of cells undergoing rolling adhesion under physiological flow conditions. Our results suggest that the ligand-binding ability of CD44 is governed by its cholesterol-dependent allocation to membrane microdomains at the cell surface. These findings provide novel insight into the regulation of T cell adhesion under blood flow.

3.2030 Expression profile and mitochondrial colocalization of Tdp1 in peripheral human tissues
Fam, H.K., Chowdhury, M.K., Walton, C., Choi, K., Boerkoel, C.F. and Hendson, G.
J. Mol. Hist., ***44(4)****, 481-494 (2013)*

Tyrosyl-DNA phosphodiesterase 1 (Tdp1) is a DNA repair enzyme that processes blocked 3′ ends of DNA breaks. Functional loss of Tdp1 causes spinocerebellar ataxia with axonal neuropathy type 1 (SCAN1). Based on the prominent cytoplasmic expression of Tdp1 in the neurons presumably affected in SCAN1, we hypothesized that Tdp1 participates in the repair of mitochondrial DNA. As a step toward testing this hypothesis, we profiled Tdp1 expression in different human tissues by immunohistochemistry and immunofluorescence respectively and determined whether Tdp1 was expressed in the cytoplasm of tissues other than the neurons. We found that Tdp1 was ubiquitously expressed and present in the cytoplasm of many cell types. Within human skeletal muscle and multiple mouse tissues, Tdp1 partially colocalized with the mitochondria. In cultured human dermal fibroblasts, Tdp1 redistributed to the cytoplasm and partially colocalized with mitochondria following oxidative stress. These studies suggest that one role of cytoplasmic Tdp1 is the repair of mitochondrial DNA lesions arising from oxidative stress.

3.2031 Visual arrestin interaction with clathrin adaptor AP-2 regulates photoreceptor survival in the vertebrate retina
Moaven, H., Koike, Y., Jao, C., Gurevich, V.V., Langen, R. and Chen, J.
PNAS, ***110(23)****, 9463-9468 (2013)*

Arrestins bind ligand-activated, phosphorylated G protein-coupled receptors (GPCRs) and terminate the activation of G proteins. Additionally, nonvisual arrestin/GPCR complex can initiate G protein-independent intracellular signals through their ability to act as scaffolds that bring other signaling molecules to the internalized GPCR. Like nonvisual arrestins, vertebrate visual arrestin (ARR1) terminates

G protein signaling from light-activated, phosphorylated GPCR, rhodopsin. Unlike nonvisual arrestins, its role as a transducer of signaling from internalized rhodopsin has not been reported in the vertebrate retina. Formation of signaling complexes with arrestins often requires recruitment of the endocytic adaptor protein, AP-2. We have previously shown that Lys296 → Glu (K296E), which is a naturally occurring rhodopsin mutation in certain humans diagnosed with autosomal dominant retinitis pigmentosa, causes toxicity through forming a stable complex with ARR1. Here we investigated whether recruitment of AP-2 by the K296E/ARR1 complex plays a role in generating the cell death signal in a transgenic mouse model of retinal degeneration. We measured the binding affinity of ARR1 for AP-2 and found that, although the affinity is much lower than that of the other arrestins, the unusually high concentration of ARR1 in rods would favor this interaction. We further demonstrate that p44, a splice variant of ARR1 that binds light-activated, phosphorylated rhodopsin but lacks the AP-2 binding motif, prevents retinal degeneration and rescues visual function in K296E mice. These results reveal a unique role of ARR1 in a G protein-independent signaling cascade in the vertebrate retina.

3.2032 **Differential effects of cocaine on histone posttranslational modifications in identified populations of striatal neurons**
Jordi, E., Heiman, M., Marion-Poll, L., Guermonprez, P., Cheng, S.K., Nairn, A.C., Greengard, P. and Girault, J-A.
*PNAS, **110(23)**, 9511-9516 (2013)*

Drugs of abuse, such as cocaine, induce changes in gene expression and epigenetic marks including alterations in histone posttranslational modifications in striatal neurons. These changes are thought to participate in physiological memory mechanisms and to be critical for long-term behavioral alterations. However, the striatum is composed of multiple cell types, including two distinct populations of medium-sized spiny neurons, and little is known concerning the cell-type specificity of epigenetic modifications. To address this question we used bacterial artificial chromosome transgenic mice, which express EGFP fused to the N-terminus of the large subunit ribosomal protein L10a driven by the D1 or D2 dopamine receptor (D1R, D2R) promoter, respectively. Fluorescence in nucleoli was used to sort nuclei from D1R- or D2R-expressing neurons and to quantify by flow cytometry the cocaine-induced changes in histone acetylation and methylation specifically in these two types of nuclei. The two populations of medium-sized spiny neurons displayed different patterns of histone modifications 15 min or 24 h after a single injection of cocaine or 24 h after seven daily injections. In particular, acetylation of histone 3 on Lys 14 and of histone 4 on Lys 5 and 12, and methylation of histone 3 on Lys 9 exhibited distinct and persistent changes in the two cell types. Our data provide insights into the differential epigenetic responses to cocaine in D1R- and D2R-positive neurons and their potential regulation, which may participate in the persistent effects of cocaine in these neurons. The method described should have general utility for studying nuclear modifications in different types of neuronal or nonneuronal cell types.

3.2033 **Identification and characterization of proteins isolated from microvesicles derived from human lung cancer pleural effusions**
Park, J.O., Choi, D-Y., Choi, D-S., Kim, H.J., Kang, J.W., Jung, J.H., Lee, J.H., Kim, J., Freeman, M.R., Lee, K.Y., Gho, Y.S. and Kim, K.P.
*Proteomics, **13(14)**, 2125-2134 (2013)*

Microvesicles (MVs, also known as exosomes, ectosomes, microparticles) are released by various cancer cells, including lung, colorectal, and prostate carcinoma cells. MVs released from tumor cells and other sources accumulate in the circulation and in pleural effusion. Although recent studies have shown that MVs play multiple roles in tumor progression, the potential pathological roles of MV in pleural effusion, and their protein composition, are still unknown. In this study, we report the first global proteomic analysis of highly purified MVs derived from human nonsmall cell lung cancer (NSCLC) pleural effusion. Using nano-LC–MS/MS following 1D SDS-PAGE separation, we identified a total of 912 MV proteins with high confidence. Three independent experiments on three patients showed that MV proteins from PE were distinct from MV obtained from other malignancies. Bioinformatics analyses of the MS data identified pathologically relevant proteins and potential diagnostic makers for NSCLC, including lung-enriched surface antigens and proteins related to epidermal growth factor receptor signaling. These findings provide new insight into the diverse functions of MVs in cancer progression and will aid in the development of novel diagnostic tools for NSCLC.

3.2034 **The Liver Connexin32 Interactome Is a Novel Plasma Membrane-Mitochondrial Signaling Nexus**
Fowler, S.L., Akins, M., Zhou, H., Figeys, D. and Bennett, A.L.
*J. Proteome Res., **12(6)**, 2597-2610 (2013)*

Connexins are the structural subunits of gap junctions and act as protein platforms for signaling complexes. Little is known about tissue-specific connexin signaling nexuses, given significant challenges associated with affinity-purifying endogenous channel complexes to the level required for interaction analyses. Here, we used multiple subcellular fractionation techniques to isolate connexin32-enriched membrane microdomains from murine liver. We show, for the first time, that connexin32 localizes to both the plasma membrane and inner mitochondrial membrane of hepatocytes. Using a combination of immunoprecipitation-high throughput mass spectrometry, reciprocal co-IP, and subcellular fractionation methodologies, we report a novel interactome validated using null mutant controls. Eighteen connexin32 interacting proteins were identified. The majority represent resident mitochondrial proteins, a minority represent plasma membrane, endoplasmic reticulum, or cytoplasmic partners. In particular, connexin32 interacts with connexin26 and the mitochondrial protein, sideroflexin-1, at the plasma membrane. Connexin32 interaction enhances connexin26 stability. Converging bioinformatic, biochemical, and confocal analyses support a role for connexin32 in transiently tethering mitochondria to connexin32-enriched plasma membrane microdomains through interaction with proteins in the outer mitochondrial membrane, including sideroflexin-1. Complex formation increases the pool of sideroflexin-1 that is present at the plasma membrane. Together, these data identify a novel plasma membrane/mitochondrial signaling nexus in the connexin32 interactome.

3.2035 Proteomic Identification of Unique Photoreceptor Disc Components Reveals the Presence of PRCD, a Protein Linked to Retinal Degeneration

Skiba, N.P:, Spencer, W.J., Salinas, R.Y., Lieu, E.C., Thompson, J.W. and Arshavsky, V.Y.
*J. Proteome Res., **12(6)**, 3010-3018 (2013)*

Visual signal transduction takes place on the surface of flat membrane vesicles called photoreceptor discs, which reside inside the light-sensitive outer segment organelle of vertebrate photoreceptor cells. Although biochemical studies have indicated that discs are built with a handful of highly specialized proteins, proteomic studies have yielded databases consisting of hundreds of entries. We addressed this controversy by employing protein correlation profiling, which allows identification of unique components of organelles that can be fractionated but not purified to absolute homogeneity. We subjected discs to sequential steps of fractionation and identified the relative amounts of proteins in each fraction by label-free quantitative mass spectrometry. This analysis demonstrated that the photoreceptor disc proteome contains only eleven components, which satisfy the hallmark criterion for being unique disc-resident components: the retention of a constant molar ratio among themselves across fractionation steps. Remarkably, one of them is PRCD, a protein whose mutations have been shown to cause blindness, yet cellular localization remained completely unknown. Identification of PRCD as a novel disc-specific protein facilitates understanding its functional role and the pathobiological significance of its mutations. Our study provides a striking example how protein correlation profiling allows a distinction between constitutive components of cellular organelles and their inevitable contaminants.

3.2036 Investigation of Polyethylenimine/DNA Polyplex Transfection to Cultured Cells Using Radiolabeling and Subcellular Fractionation Methods

Shi, J., Chou, B., Choi, J.L., Ta, A.L. and Pun, S.H.
*Mol. Pharmaceutics, **10(6)**, 2145-2156 (2013)*

Quantitative analysis of the intracellular trafficking of nonviral vectors provides critical information that can guide the rational design of improved cationic systems for gene delivery. Subcellular fractionation methods, combined with radiolabeling, produce quantitative measurements of the intracellular trafficking of nonviral vectors and the therapeutic payload. In this work, differential and density-gradient centrifugation techniques were used to determine the intracellular distribution of radiolabeled 25 kDa branched polyethylenimine (bPEI)/plasmid DNA complexes ("polyplexes") in HeLa cells over time. By differential centrifugation, [^{14}C]bPEI was found mostly in the lighter fractions whereas [^{3}H]DNA was found mostly in the heavier fractions. A majority of the intracellular polymer (~60%) and DNA (~90%) were found in the nuclear fraction. Polymer and DNA also differed in their distribution to heavier and denser organelles (lysosomes, mitochondria) in density-gradient centrifugation studies. An unexpected finding from this study was that between 18 and 50% of the DNA applied to the cells became cell-associated (either with the cell membrane and/or internalized), while only 1–6% of the polymer did so, resulting in an effective N/P ratio of less than 1. These results suggest that a significant amount of cationic polymer is dissociated from the DNA cargo early on in the transfection process.

3.2037 **Meigo governs dendrite targeting specificity by modulating Ephrin level and N-glycosylation**
Sekine, S.U., haraguchi, S., Chao, K., kato, T., Luo, L., Miura, M. and Chihara, T.
Nature Neurosci., ***16(6)****, 683-691 (2013)*

Neural circuit assembly requires precise dendrite and axon targeting. We identified an evolutionarily conserved endoplasmic reticulum (ER) protein, Meigo, from a mosaic genetic screen in *Drosophila melanogaster*. Meigo was cell-autonomously required in olfactory receptor neurons and projection neurons to target their axons and dendrites to the lateral antennal lobe and to refine projection neuron dendrites into individual glomeruli. Loss of Meigo induced an unfolded protein response and reduced the amount of neuronal cell surface proteins, including Ephrin. Ephrin overexpression specifically suppressed the projection neuron dendrite refinement defect present in *meigo* mutant flies, and *ephrin* knockdown caused a similar projection neuron dendrite refinement defect. Meigo positively regulated the level of Ephrin *N*-glycosylation, which was required for its optimal function *in vivo*. Thus, Meigo, an ER-resident protein, governs neuronal targeting specificity by regulating ER folding capacity and protein *N*-glycosylation. Furthermore, Ephrin appears to be an important substrate that mediates Meigo's function in refinement of glomerular targeting.

3.2038 **Mechanisms Responsible for the Compositional Heterogeneity of Nascent High Density Lipoprotein**
Lund-Katz, S., Lyssenko, N.N., Nickel, M., Nguyen, D., Chetty, P.S., Weibel, G. and Philips, M.C.
J. Biol. Chem., ***288(32)****, 23150-23160 (2013)*

Apolipoprotein (apo) A-I-containing nascent HDL particles produced by the ATP binding cassette transporter A1 have different sizes and compositions. To understand the molecular basis for this heterogeneity, the HDL particles produced by apoA-I-mediated solubilization of phospholipid (PL)/free (unesterified) cholesterol (FC) bilayer membranes in cell and cell-free systems are compared. Incubation of apoA-I with ATP binding cassette transporter A1-expressing baby hamster kidney cells leads to formation of two populations of FC-containing discoidal nascent HDL particles. The larger 11-nm diameter particles are highly FC-enriched (FC/PL = 1.2/1 mol/mol) relative to the smaller 8 nm particles and the cell plasma membrane (FC/PL = 0.4/1). ApoA-I-mediated spontaneous solubilization of either multilamellar or unilamellar vesicles made of a membrane-PL mixture and FC yields discoidal HDL particles with diameters in the range 9–17 nm and, as found with the cell system, the larger particles are relatively enriched in FC despite the fact that all particles are created by solubilization of a common FC/PL membrane domain. The size-dependent distribution of FC among HDL particles is due to varying amounts of PL being sequestered in a boundary layer by interaction with apoA-I at the disc edge. The presence of a relatively large boundary layer in smaller discoidal HDL promotes preferential distribution of phosphatidylserine to such particles. However, phosphatidylcholine and sphingomyelin which are the primary PL constituents of nascent HDL do not exhibit selective incorporation into HDL discs of different sizes. This understanding of the mechanisms responsible for the heterogeneity in lipid composition of nascent HDL particles may provide a basis for selecting subspecies with preferred cardio-protective properties.

3.2039 **The Us2 Gene Product of Herpes Simplex Virus 2 Is a Membrane-Associated Ubiquitin-Interacting Protein**
Kang, M-H., Roy, B.B., Finnen, R.L., Le Sage, V., Johnston, S.M., Zhang, H and Banfield, B.W.
J. Virol., ***87(17)****, 9590-9603 (2013)*

The *Us2* gene encodes a tegument protein that is conserved in most members of the *Alphaherpesvirinae*. Previous studies on the pseudorabies virus (PRV) Us2 ortholog indicated that it is prenylated, associates with membranes, and spatially regulates the enzymatic activity of the MAP (mitogen-activated protein) kinase ERK (extracellular signal-related kinase) through direct binding and sequestration of ERK at the cytoplasmic face of the plasma membrane. Here we present an analysis of the herpes simplex virus 2 (HSV-2) Us2 ortholog and demonstrate that, like PRV Us2, HSV-2 Us2 is a virion component and that, unlike PRV Us2, it does not interact with ERK in yeast two-hybrid assays. HSV-2 Us2 lacks prenylation signals and other canonical membrane-targeting motifs yet is tightly associated with detergent-insoluble membranes and localizes predominantly to recycling endosomes. Experiments to identify cellular proteins that facilitate HSV-2 Us2 membrane association were inconclusive; however, these studies led to the identification of HSV-2 Us2 as a ubiquitin-interacting protein, providing new insight into the functions of HSV-2 Us2.

3.2040 The Cytosolic Adaptor AP-1A Is Essential for the Trafficking and Function of Niemann-Pick Type C Proteins

Poirier, S., Mayer, G., Murphy, S.R., Garver, W.S., chang, T.Y., Schu, P. and Seidah, N.G.
Traffic, ***14(4)***, *458-469 (2013)*

Niemann-Pick type C (NPC) disease is a fatal neurodegenerative disorder characterized by over-accumulation of low-density lipoprotein-derived cholesterol and glycosphingolipids in late endosomes/lysosomes (LE/L) throughout the body. Human mutations in either *NPC1* or *NPC2* genes have been directly associated with impaired cholesterol efflux from LE/L. Independent from its role in cholesterol homeostasis and its NPC2 partner, NPC1 was unexpectedly identified as a critical player controlling intracellular entry of filoviruses such as Ebola. In this study, a yeast three-hybrid system revealed that the NPC1 cytoplasmic tail directly interacts with the clathrin adaptor protein AP-1 *via* its acidic/di-leucine motif. Consequently, a nonfunctional AP-1A cytosolic complex resulted in a typical NPC-like phenotype mainly due to a direct impairment of NPC1 trafficking to LE/L and a partial secretion of NPC2. Furthermore, the mislocalization of NPC1 was not due to cholesterol accumulation in LE/L, as it was not rescued upon treatment with Mβ-cyclodextrin, which almost completely eliminated intracellular free cholesterol. Our cumulative data demonstrate that the cytosolic clathrin adaptor AP-1A is essential for the lysosomal targeting and function of NPC1 and NPC2.

3.2041 Differential Endosomal Sorting of a Novel P2Y12 Purinoreceptor Mutant

Cunningham, M.R., Nisar, S.P., Cooke, A.E., Emery, E.D. and Mundell, S.J.
Traffic, ***14(5)***, *585-598 (2013)*

$P2Y_{12}$ receptor internalization and recycling play an essential role in ADP-induced platelet activation. Recently, we identified a patient with a mild bleeding disorder carrying a heterozygous mutation of $P2Y_{12}$ (P341A) whose $P2Y_{12}$ receptor recycling was significantly compromised. Using human cell line models, we identified key proteins regulating wild-type (WT) $P2Y_{12}$ recycling and investigated $P2Y_{12}$-P341A receptor traffic. Treatment with ADP resulted in delayed Rab5-dependent internalization of P341A when compared with WT $P2Y_{12}$. While WT $P2Y_{12}$ rapidly recycled back to the membrane via Rab4 and Rab11 recycling pathways, limited P341A recycling was observed, which relied upon Rab11 activity. Although minimal receptor degradation was evident, P341A was localized in Rab7-positive endosomes with considerable agonist-dependent accumulation in the *trans*-Golgi network (TGN). Rab7 activity is known to facilitate recruitment of retromer complex proteins to endosomes to transport cargo to the TGN. Here, we identified that P341A colocalized with Vps26; depletion of which blocked limited recycling and promoted receptor degradation. This study has identified key points of divergence in the endocytic traffic of P341A versus WT-$P2Y_{12}$. Given that these pathways are retained in human platelets, this research helps define the molecular mechanisms regulating $P2Y_{12}$ receptor traffic and explain the compromised receptor function in the platelets of the $P2Y_{12}$-P341A-expressing patient.

3.2042 Association of the Vaccinia Virus A11 Protein with the Endoplasmic Reticulum and Crescent Precursors of Immature Virions

Maruri-Avidal, L., Weisberg, A.S. and Moss, B.
J. Virol., ***87(18)***, *10195-10206 (2013)*

The apparent *de novo* formation of viral membranes within cytoplasmic factories is a mysterious, poorly understood first step in poxvirus morphogenesis. Genetic studies identified several viral proteins essential for membrane formation and the assembly of immature virus particles. Their repression results in abortive replication with the accumulation of dense masses of viroplasm. In the present study, we further characterized one of these proteins, A11, and investigated its association with cellular and viral membranes under normal and abortive replication conditions. We discovered that A11 colocalized in cytoplasmic factories with the endoplasmic reticulum (ER) and L2, another viral protein required for morphogenesis. Confocal microscopy and subcellular fractionation indicated that A11 was not membrane associated in uninfected cells, whereas L2 still colocalized with the ER. Cell-free transcription and translation experiments indicated that both A11 and L2 are tail-anchored proteins that associate posttranslationally with membranes and likely require specific cytoplasmic targeting chaperones. Transmission electron microscopy indicated that A11, like L2, associated with crescent membranes and immature virions during normal infection and with vesicles and tubules near masses of dense viroplasm during abortive infection in the absence of the A17 or A14 protein component of viral membranes. When the synthesis of A11 was repressed, "empty" immature-virion-like structures formed in addition to masses of viroplasm. The immature-virion-like structures were labeled with antibodies to A17 and to the D13 scaffold protein and

were closely associated with calnexin-labeled ER. These studies revealed similarities and differences between A11 and L2, both of which may be involved in the recruitment of the ER for virus assembly.

3.2043 Suppression of Aβ toxicity by puromycin-sensitive aminopeptidase is independent of its proteolytic activity

Kruppa, A.J., Ott, S., Chandraratna, D.S., Irving, J.A., Page, R.M., Speretta, E., Seto, T., Camargo, L:M., Marciniak, S.J., Lomas, D.A. and Crowther, D.C.
*Biochim. Biophys. Acta, **1832**, 2115-2126 (2013)*

The accumulation of β-amyloid (Aβ) peptide in the brain is one of the pathological hallmarks of Alzheimer's disease and is thought to be of primary aetiological significance. In an unbiased genetic screen, we identified puromycin-sensitive aminopeptidase (PSA) as a potent suppressor of Aβ toxicity in a *Drosophila* model system. We established that coexpression of *Drosophila* PSA (dPSA) in the flies' brains improved their lifespan, protected against locomotor deficits, and reduced brain Aβ levels by clearing the Aβ plaque-like deposits. However, confocal microscopy and subcellular fractionation of amyloid-expressing 7PA2 cells demonstrated that PSA localizes to the cytoplasm. Therefore, PSA and Aβ are unlikely to be in the same cellular compartment; moreover, when we artificially placed them in the same compartment in flies, we could not detect a direct epistatic interaction. The consequent hypothesis that PSA's suppression of Aβ toxicity is indirect was supported by the finding that Aβ is not a proteolytic substrate for PSA *in vitro*. Furthermore, we showed that the enzymatic activity of PSA is not required for rescuing Aβ toxicity in neuronal SH-SY5Y cells. We investigated whether the stimulation of autophagy by PSA was responsible for these protective effects. However PSA's promotion of autophagosome fusion with lysosomes required proteolytic activity and so its effect on autophagy is not identical to its protection against Aβ toxicity.

3.2044 Plasma membrane Pdia3 and VDR interact to elicit rapid responses to 1α,25(OH)2D3

Chen, J., Doroudi, M., Cheung, J., Grozier, A.L., Schweartz, Z. and Boyan, B.D.
*Cellular Signalling, **25**, 2362-2373 (2013)*

1α,25-Dihydroxyvitamin D3 (1α,25$(OH)_2D_3$) regulates osteoblasts through genomic and rapid membrane-mediated responses. Here we examined the interaction of protein disulfide isomerase family A, member 3 (Pdia3) and the traditional vitamin D receptor (VDR) in plasma membrane-associated responses to 1α,25$(OH)_2D_3$. We found that Pdia3 co-localized with VDR and the caveolae scaffolding protein, caveolin-1 on the surface of MC3T3-E1 osteoblasts. Immunoprecipitation showed that both Pdia3 and VDR interacted with caveolin-1. Pdia3 further interacted with phospholipase A2 activating protein (PLAA), whereas VDR interacted with c-Src. 1α,25$(OH)_2D_3$ changed the interactions and transport of the two receptors and rapidly activated phospholipase A2 (PLA2) and c-Src. Silencing either receptor or caveolin-1 inhibited both PLA2 and c-Src, indicating that the two receptors function interdependently. These two receptor dependent rapid responses to 1α,25$(OH)_2D_3$ regulated gene expression, proliferation and apoptosis of MC3T3-E1 cells. These data demonstrate the importance of both receptors and caveolin-1 in mediating membrane responses to 1α,25$(OH)_2D_3$ and subsequently regulating osteoblast biology.

3.2045 A simple methodology to assess endolysosomal protease activity involved in antigen processing in human primary cells

Vaithikingam, A., Lai, N.Y., Duong, E., Boucau, J., Xu, Y., Shimada, M., Gabdhi, M. and Le Gall, S.
*BMC Cell Biol., **14**:35 (2013)*

Background
Endolysosomes play a key role in maintaining the homeostasis of the cell. They are made of a complex set of proteins that degrade lipids, proteins and sugars. Studies involving endolysosome contribution to cellular functions such as MHC class I and II epitope production have used recombinant endolysosomal proteins, knockout mice that lack one of the enzymes or purified organelles from human tissue. Each of these approaches has some caveats in analyzing endolysosomal enzyme functions.
Results
In this study, we have developed a simple methodology to assess endolysosomal protease activity. By varying the pH in crude lysate from human peripheral blood mononuclear cells (PBMCs), we documented increased endolysosomal cathepsin activity in acidic conditions. Using this new method, we showed that the degradation of HIV peptides in low pH extracts analyzed by mass spectrometry followed similar kinetics and degradation patterns as those performed with purified endolysosomes.

Conclusion
By using crude lysate in the place of purified organelles this method will be a quick and useful tool to assess endolysosomal protease activities in primary cells of limited availability. This quick method will especially be useful to screen peptide susceptibility to degradation in endolysosomal compartments for antigen processing studies, following which detailed analysis using purified organelles may be used to study specific peptides.

3.2046 Certain Strongylocentrotus purpuratus sperm mitochondrial proteins co-purify with low density detergent-insoluble membranes and are PKA or PKC-substrates possibly involved in sperm motility regulation
Loza-Huerta, A., Vera-Estrella, R., Darszon, A. and Beltran, C.
*Biochim. Biophys. Acta, **1830**, 5305-5315 (2013)*

Background
Sea urchin sperm motility is regulated by Speract, a sperm-activating peptide (SAP) secreted from the outer egg coat. Upon binding to its receptor in the sperm flagellum, Speract induces a series of ionic and metabolic changes in *Strongylocentrotus purpuratus* spermatozoa that regulate their motility. Among these events, protein phosphorylation is one of the most relevant and evidence indicates that some proteins of the Speract signaling cascade localize in low density detergent-insoluble membranes (LD-DIM).
Methods
LD-DIM-derived proteins from immotile, motile or Speract-stimulated *S. purpuratus* sperm were resolved in 2-D gels and the PKA and PKC substrates detected with specific antibodies were identified by LC–MS/MS.
Results
Differential PKA and PKC substrate phosphorylation levels among the LD-DIM isolated from sperm in different motility conditions were found and identified by mass spectrometry as: ATP synthase, creatine kinase, NADH dehydrogenase (ubiquinone) flavoprotein 2, succinyl-CoA ligase and the voltage-dependent anion channel 2 (VDAC2), which are mitochondrial proteins, as well as, the cAMP-dependent protein kinase type II regulatory (PKA RII) subunit, Tubulin β chain and Actin Cy I changed their phosphorylation state.
Conclusions
Some mitochondrial proteins regulated by PKA or PKC may influence sea urchin sperm motility.
General significance
The fact that a high percentage (66%) of the PKA or PKC substrates identified in LD-DIM are mitochondrial proteins suggests that the phosphorylation of these proteins modulates sea urchin sperm motility via Speract stimulation by providing sufficient energy to sperm physiology. Those mitochondrial proteins are indeed PKA- or PKC-substrates in the sea urchin spermatozoa.

3.2047 CD147, CD44, and the Epidermal Growth Factor Receptor (EGFR) Signaling Pathway Cooperate to Regulate Breast Epithelial Cell Invasiveness
Grass, G.D., Tolliver, L.B., Bratoeva, M. and Toole, B.P.
*J. Biol. Chem., **288(36)**, 26089-26104 (2013)*

The immunoglobulin superfamily glycoprotein CD147 (emmprin; basigin) is associated with an invasive phenotype in various types of cancers, including malignant breast cancer. We showed recently that up-regulation of CD147 in non-transformed, non-invasive breast epithelial cells is sufficient to induce an invasive phenotype characterized by membrane type-1 matrix metalloproteinase (MT1-MMP)-dependent invadopodia activity (Grass, G. D., Bratoeva, M., and Toole, B. P. (2012) Regulation of invadopodia formation and activity by CD147. *J. Cell Sci.* 125, 777–788). Here we found that CD147 induces breast epithelial cell invasiveness by promoting epidermal growth factor receptor (EGFR)-Ras-ERK signaling in a manner dependent on hyaluronan-CD44 interaction. Furthermore, CD147 promotes assembly of signaling complexes containing CD147, CD44, and EGFR in lipid raftlike domains. We also found that oncogenic Ras regulates CD147 expression, hyaluronan synthesis, and formation of CD147-CD44-EGFR complexes, thus forming a positive feedback loop that may amplify invasiveness. Last, we showed that malignant breast cancer cells are heterogeneous in their expression of surface-associated CD147 and that high levels of membrane CD147 correlate with cell surface EGFR and CD44 levels, activated EGFR and ERK1, and activated invadopodia. Future studies should evaluate CD147 as a potential therapeutic target and disease stratification marker in breast cancer.

3.2048 Myosin-1c regulates the dynamic stability of E-cadherin–based cell–cell contacts in polarized Madin–Darby canine kidney cells
Tokuo, H. and Coluccio, L.M.
*Mol. Biol. Cell, **24**, 2820-2832 (2013)*

Cooperation between cadherins and the actin cytoskeleton controls the formation and maintenance of cell–cell adhesions in epithelia. We find that the molecular motor protein myosin-1c (Myo1c) regulates the dynamic stability of E-cadherin–based cell–cell contacts. In Myo1c-depleted Madin–Darby canine kidney cells, E-cadherin localization was disorganized and lateral membranes appeared less vertical with convoluted edges versus control cells. In polarized monolayers, Myo1c-knockdown (KD) cells were more sensitive to reduced calcium concentration. Myo1c separated in the same plasma membrane fractions as E-cadherin, and Myo1c KD caused a significant reduction in the amount of E-cadherin recovered in one peak fraction. Expression of green fluorescent protein (GFP)–Myo1c mutants revealed that the phosphatidylinositol-4,5-bisphosphate–binding site is necessary for its localization to cell–cell adhesions, and fluorescence recovery after photobleaching assays with GFP-Myo1c mutants revealed that motor function was important for Myo1c dynamics at these sites. At 18°C, which inhibits vesicle recycling, Myo1c-KD cells accumulated more E-cadherin–positive vesicles in their cytoplasm, suggesting that Myo1c affects E-cadherin endocytosis. Studies with photoactivatable GFP–E-cadherin showed that Myo1c KD reduced the stability of E-cadherin at cell–cell adhesions. We conclude that Myo1c stabilizes E-cadherin at adherens junctions in polarized epithelial cells and that the motor function and ability of Myo1c to bind membrane are critical.

3.2049 Regulation of pre-fusion events: recruitment of M-cadherin to microrafts organized at fusion-competent sites of myogenic cells
Mukai, A. and Hashimoto, N.
*BMC Cell Biol., **14**:37 (2013)*

Background
Previous research indicates that the membrane ruffles and leading edge of lamellipodia of myogenic cells contain presumptive fusion sites. A micrometer-sized lipid raft (microraft) is organized at the presumptive fusion site of mouse myogenic cells in a cell-contact independent way and serves as a platform tethering adhesion proteins that are relevant to cell fusion. However, the mechanisms underlying recruitment of adhesion proteins to lipid rafts and microraft organization remain unknown.
Results
Here we show that small G-protein Rac1 was required for microraft organization and subsequent cell fusion. However, Rac1 activity was unnecessary for recruitment of M-cadherin to lipid rafts. We found that p120 catenin (p120) binds to M-cadherin exclusively in lipid rafts of differentiating myogenic cells. The Src kinase inhibitor SU6656 prevented p120 binding to M-cadherin and their recruitment to lipid rafts, then suppressed microraft organization, membrane ruffling, and myogenic cell fusion. Suppression of membrane ruffling in SU6656-treated cells was partially restored by pretreatment with the protein tyrosine phosphatase inhibitor vanadate. The present analyses using an antibody to tyrosine phosphorylated p120 suggest that Src family kinases play a role in binding of p120 to M-cadherin and the recruitment of M-cadherin to lipid rafts through phosphorylation of putative substrates other than p120.
Conclusions
The present study showed that the procedure establishing fusion-competent sites consists of two sequential events: recruitment of adhesion complexes to lipid rafts and organization of microrafts. The recruitment of M-cadherin to lipid rafts depended on interaction with p120 catenin, whereas the organization of microrafts was controlled by a small G protein, Rac1.

3.2050 Cetuximab enhances TRAIL-induced gastric cancer cell apoptosis by promoting DISC formation in lipid rafts
Xu, L., Hu, X., Qu, X., Hou, K., Zheng, H. and Liu, Y.
*Biochem. Biophys. Res. Comm., **439**, 285-290 (2013)*

TRAIL is a member of the tumor necrosis factor family that selectively induces cancer cell apoptosis. However, gastric cancer cells are insensitive to TRAIL. Our and others studies showed that the inhibition of EGFR pathway activation could increase the sensitivity of TRAIL in cancer cells. But the detailed mechanism is not fully understood. In the present study, compared with TRAIL or cetuximab (an anti-EGFR monoclonal antibody) alone, treatment with the TRAIL/cetuximab combination significantly promoted death receptor 4 (DR4) clustering as well as the translocation of both DR4 and Fas-associated

death domain-containing protein (FADD) into lipid rafts. This in turn resulted in caspase-8 cleavage and the formation of the death-inducing signaling complex (DISC) in these lipid rafts. Cholesterol-depletion with methyl- β -cyclodextrin partially prevented DR4 clustering and DISC formation, and thus partially reversed apoptosis induced by the TRAIL/cetuximab dual treatment. These results indicate that cetuximab increases TRAIL-induced gastric cancer cell apoptosis at least partially through the promotion of DISC formation in lipid rafts.

3.2051 Ubiquitination of the glycosomal matrix protein receptor PEX5 in Trypanosoma brucei by PEX4 displays novel features

Gualdron-Lopez, M., Chevalier, N., Van Der Smissen, P., Courtoy, P.J., Rigden, D.J. and Michels, P.A.M.
*Biochim. Biophys. Acta, **1833**, 3076-3092 (2013)*

Trypanosomatids contain peroxisome-like organelles called glycosomes. Peroxisomal biogenesis involves a cytosolic receptor, PEX5, which, after its insertion into the organellar membrane, delivers proteins to the matrix. In yeasts and mammalian cells, transient PEX5 monoubiquitination at the membrane serves as the signal for its retrieval from the organelle for re-use. When its recycling is impaired, PEX5 is polyubiquitinated for proteasomal degradation. Stably monoubiquitinated TbPEX5 was detected in cytosolic fractions of *Trypanosoma brucei*, indicative for its role as physiological intermediate in receptor recycling. This modification's resistance to dithiothreitol suggests ubiquitin conjugation of a lysine residue. *T. brucei* PEX4, the functional homologue of the ubiquitin-conjugating (UBC) enzyme responsible for PEX5 monoubiquitination in yeast, was identified. It is associated with the cytosolic face of the glycosomal membrane, probably anchored by an identified putative TbPEX22. The involvement of TbPEX4 in TbPEX5 ubiquitination was demonstrated using procyclic *ΔPEX4* trypanosomes. Surprisingly, glycosomal matrix protein import was only mildly affected in this mutant. Since other UBC homologues were upregulated, it might be possible that these have partially rescued PEX4's function in PEX5 ubiquitination. In addition, the altered expression of UBCs, notably of candidates involved in cell-cycle control, could be responsible for observed morphological and motility defects of the *ΔPEX4* mutant.

3.2052 Cavin-3 dictates the balance between ERK and Akt signaling

Victor J Hernandez, Jian Weng, Peter Ly, Shanica Pompey, Hongyun Dong, Lopa Mishra, Margaret Schwarz, Richard GW Anderson, and Peter Michaely
*eLife, **2**, e00905 (2013)*

Cavin-3 is a tumor suppressor protein of unknown function. Using both in vivo and in vitro approaches, we show that cavin-3 dictates the balance between ERK and Akt signaling. Loss of cavin-3 increases Akt signaling at the expense of ERK, while gain of cavin-3 increases ERK signaling at the expense Akt. Cavin-3 facilitates signal transduction to ERK by anchoring caveolae to the membrane skeleton of the plasma membrane via myosin-1c. Caveolae are lipid raft specializations that contain an ERK activation module and loss of the cavin-3 linkage reduces the abundance of caveolae, thereby separating this ERK activation module from signaling receptors. Loss of cavin-3 promotes Akt signaling through suppression of EGR1 and PTEN. The in vitro consequences of the loss of cavin-3 include induction of Warburg metabolism (aerobic glycolysis), accelerated cell proliferation, and resistance to apoptosis. The in vivo consequences of cavin-3 knockout are increased lactate production and cachexia.

3.2053 Leucine Carboxyl Methyltransferase 1 (LCMT1)-dependent Methylation Regulates the Association of Protein Phosphatase 2A and Tau Protein with Plasma Membrane Microdomains in Neuroblastoma Cells

Jean-Marie Sontag, Viyada Nunbhakdi-Craig, and Estelle Sontag
*J. Biol. Chem., **288(38)**, 27396-27405 (2013)*

Down-regulation of protein phosphatase 2A (PP2A) methylation occurs in Alzheimer disease (AD). However, the regulation of PP2A methylation remains poorly understood. We have reported that altered leucine carboxyl methyltransferase (LCMT1)-dependent PP2A methylation is associated with down-regulation of PP2A holoenzymes containing the Bα subunit (PP2A/Bα) and subsequent accumulation of phosphorylated Tau in N2a cells, *in vivo* and in AD. Here, we show that pools of LCMT1, methylated PP2A, and PP2A/Bα are co-enriched in cholesterol-rich plasma membrane microdomains/rafts purified from N2a cells. In contrast, demethylated PP2A is preferentially distributed in non-rafts wherein small amounts of the PP2A methylesterase PME-1 are exclusively present. A methylation-incompetent PP2A

mutant is excluded from rafts. Enhanced methylation of PP2A promotes the association of PP2A and Tau with the plasma membrane. Altered PP2A methylation following expression of a catalytically inactive LCMT1 mutant, knockdown of LCMT1, or alterations in one-carbon metabolism all result in a loss of plasma membrane-associated PP2A and Tau in N2a cells. This correlates with accumulation of soluble phosphorylated Tau, a hallmark of AD and other tauopathies. Thus, our findings reveal a distinct compartmentalization of PP2A and PP2A regulatory enzymes in plasma membrane microdomains and identify a novel methylation-dependent mechanism involved in modulating the targeting of PP2A, and its substrate Tau, to the plasma membrane. We propose that alterations in the membrane localization of PP2A and Tau following down-regulation of LCMT1 may lead to PP2A and Tau dysfunction in AD.

3.2054 **Proteomics, transcriptomics and lipidomics of exosomes and ectosomes**
Choi, D-S., Kim, D-K., Kim, Y-K. and Gho, Y.S.
Proteomics, ***13(10-11)****, 1554-1571 (2013)*

Mammalian cells secrete two types of extracellular vesicles either constitutively or in a regulated manner: exosomes (50–100 nm in diameter) released from the intracellular compartment and ectosomes (also called microvesicles, 100–1000 nm in diameter) shed directly from the plasma membrane. Extracellular vesicles are bilayered proteolipids enriched with proteins, mRNAs, microRNAs, and lipids. In recent years, much data have been collected regarding the specific components of extracellular vesicles from various cell types and body fluids using proteomic, transcriptomic, and lipidomic methods. These studies have revealed that extracellular vesicles harbor specific types of proteins, mRNAs, miRNAs, and lipids rather than random cellular components. These results provide valuable information on the molecular mechanisms involved in vesicular cargo-sorting and biogenesis. Furthermore, studies of these complex extracellular organelles have facilitated conceptual advancements in the field of intercellular communication under physiological and pathological conditions as well as for disease-specific biomarker discovery. This review focuses on the proteomic, transcriptomic, and lipidomic profiles of extracellular vesicles, and will briefly summarize recent advances in the biology, function, and diagnostic potential of vesicle-specific components.

3.2055 **Tumor-derived exosomes and microvesicles in head and neck cancer: Implications for tumor biology and biomarker discovery**
Principe, S., Hui, A.B-Y., Bruce, J., Sinha, A., Liu, F-F. and Kislinger, T.
Proteomics, ***13(10-11)****, 1608-1623 (2013)*

Exosomes and microvesicles (MVs) are nanometer-sized, membranous vesicles secreted from many cell types into their surrounding extracellular space and into body fluids. These two classes of extracellular vesicles are regarded as a novel mechanism through which cancer cells, including virally infected cancer cells, regulate their micro-environment via the horizontal transfer of bioactive molecules: proteins, lipids, and nucleic acids (DNA, mRNA, micro-RNAs; oncogenic cargo hence often referred to as oncosomes). In head and neck cancer (HNC), exosomes and MVs have been described in Epstein Barr Virus (EBV)-associated nasopharyngeal cancer (NPC), as well as being positively correlated with oral squamous cell carcinoma (OSCC) progression. It has therefore been suggested that HNC-derived vesicles could represent a useful source for biomarker discovery, enriched in tumor antigens and cargo; hence fundamentally important for cancer progression. This current review offers an overall perspective on the roles of exosomes and MVs in HNC biology, focusing on EBV-associated NPC and OSCC. We also highlight the importance of saliva as a proximal and easily accessible bio-fluid for HNC detection, and propose that salivary vesicles might serve as an alternative model in the discovery of novel HNC biomarkers.

3.2056 **Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components**
Ji, H., Greening, D.W., barnes, T.W., Lim, J.W., Tauro, B.J., Rai, A., Xu, R., Adda, C., Mathivanan, S., Zhao, W., Xue, Y., Xu, T., Zhu, H-J. and Simpson, R.J.
Proteomics, ***13(10-11)****, 1672-1686 (2013)*

Exosomes are small extracellular 40–100 nm diameter membrane vesicles of late endosomal origin that can mediate intercellular transfer of RNAs and proteins to assist premetastatic niche formation. Using primary (SW480) and metastatic (SW620) human isogenic colorectal cancer cell lines we compared exosome protein profiles to yield valuable insights into metastatic factors and signaling molecules fundamental to tumor progression. Exosomes purified using OptiPrep™ density gradient fractionation

were 40–100 nm in diameter, were of a buoyant density ~1.09 g/mL, and displayed stereotypic exosomal markers TSG101, Alix, and CD63. A major finding was the selective enrichment of metastatic factors (MET, S100A8, S100A9, TNC), signal transduction molecules (EFNB2, JAG1, SRC, TNIK), and lipid raft and lipid raft-associated components (CAV1, FLOT1, FLOT2, PROM1) in exosomes derived from metastatic SW620 cells. Additionally, using cryo-electron microscopy, ultrastructural components in exosomes were identified. A key finding of this study was the detection and colocalization of protein complexes EPCAM-CLDN7 and TNIK-RAP2A in colorectal cancer cell exosomes. The selective enrichment of metastatic factors and signaling pathway components in metastatic colon cancer cell-derived exosomes contributes to our understanding of the cross-talk between tumor and stromal cells in the tumor microenvironment.

3.2057 Interplay between Clathrin and Rab5 Controls the Early Phagocytic Trafficking and Intracellular Survival of Brucella abortus within HeLa cells

Lee, J.J., Kim, D.G., Kim, D.H., Simborio, H.L., Min, W., Lee, H.J., Her, M., Jung, S.C., Watarai, M. and Kim, S.

J. Biol. Chem., ***288(39)***, *28049-28057 (2013)*

Lipid raft-associated clathrin is essential for host-pathogen interactions during infection. *Brucella abortus* is an intracellular pathogen that circumvents host defenses, but little is known about the precise infection mechanisms that involve interaction with lipid raft-associated mediators. The aim of this study was to elucidate the clathrin-mediated phagocytic mechanisms of *B. abortus*. The clathrin dependence of *B. abortus* infection in HeLa cells was investigated using an infection assay and immunofluorescence microscopy. The redistribution of clathrin in the membrane and in phagosomes was investigated using sucrose gradient fractionation of lipid rafts and the isolation of *B. abortus*-containing vacuoles, respectively. Clathrin and dynamin were concentrated into lipid rafts during *B. abortus* infection, and the entry and intracellular survival of *B. abortus* within HeLa cells were abrogated by clathrin inhibition. Clathrin disruption decreased actin polymerization and the colocalization of *B. abortus*-containing vacuoles with clathrin and Rab5 but not lysosome-associated membrane protein 1 (LAMP-1). Thus, our data demonstrate that clathrin plays a fundamental role in the entry and intracellular survival of *B. abortus* via interaction with lipid rafts and actin rearrangement. This process facilitates the early intracellular trafficking of *B. abortus* to safe replicative vacuoles.

3.2058 Palmitoylation is the switch that assigns calnexin to quality control or ER Ca2+ signaling

Lynes, E.M., Raturi, A., Shenkman, M., Sandoval, C.O., Yap, M.C., Wu, J., Janowicz, A., Myhill, N., Benson, M.D., Campbell, R.E., Berthiaume, L.G., Lederkremer, G.Z. and Simmen, T.

J. Cell Science, ***126(17)***, *3893-3903 (2013)*

The palmitoylation of calnexin serves to enrich calnexin on the mitochondria-associated membrane (MAM). Given a lack of information on the significance of this finding, we have investigated how this endoplasmic reticulum (ER)-internal sorting signal affects the functions of calnexin. Our results demonstrate that palmitoylated calnexin interacts with sarcoendoplasmic reticulum (SR) Ca^{2+} transport ATPase (SERCA) 2b and that this interaction determines ER Ca^{2+} content and the regulation of ER–mitochondria Ca^{2+} crosstalk. In contrast, non-palmitoylated calnexin interacts with the oxidoreductase ERp57 and performs its well-known function in quality control. Interestingly, our results also show that calnexin palmitoylation is an ER-stress-dependent mechanism. Following a short-term ER stress, calnexin quickly becomes less palmitoylated, which shifts its function from the regulation of Ca^{2+} signaling towards chaperoning and quality control of known substrates. These changes also correlate with a preferential distribution of calnexin to the MAM under resting conditions, or the rough ER and ER quality control compartment (ERQC) following ER stress. Our results have therefore identified the switch that assigns calnexin either to Ca^{2+} signaling or to protein chaperoning.

3.2059 Green tea phenolics inhibit butyrate-induced differentiation of colon cancer cells by interacting with monocarboxylate transporter 1

Sanchez-Tena, S., Vizan, P., Dudeja, P.K., Centelles, J.J. and Casante, M.

Biochim. Biophys. Acta, ***1832***, *2264-2270 (2013)*

Diet has a significant impact on colorectal cancer and both dietary fiber and plant-derived compounds have been independently shown to be inversely related to colon cancer risk. Butyrate (NaB), one of the principal products of dietary fiber fermentation, induces differentiation of colon cancer cell lines by inhibiting histone deacetylases (HDACs). On the other hand, (−)-epicatechin (EC) and (−)-epigallocatechin gallate

(EGCG), two abundant phenolic compounds of green tea, have been shown to exhibit antitumoral properties. In this study we used colon cancer cell lines to study the cellular and molecular events that take place during co-treatment with NaB, EC and EGCG. We found that (i) polyphenols EC and EGCG fail to induce differentiation of colon adenocarcinoma cell lines; (ii) polyphenols EC and EGCG reduce NaB-induced differentiation; (iii) the effect of the polyphenols is specific for NaB, since differentiation induced by other agents, such as trichostatin A (TSA), was unaltered upon EC and EGCG treatment, and (iv) is independent of the HDAC inhibitory activity of NaB. Also, (v) polyphenols partially reduce cellular NaB; and (vi) on a molecular level, reduction of cellular NaB uptake by polyphenols is achieved by impairing the capacity of NaB to relocalize its own transporter (monocarboxylate transporter 1, MCT1) in the plasma membrane. Our findings suggest that beneficial effects of NaB on colorectal cancer may be reduced by green tea phenolic supplementation. This valuable information should be of assistance in choosing a rational design for more effective diet-driven therapeutic interventions in the prevention or treatment of colorectal cancer.

3.2060 An abundant LEA protein in the anhydrobiotic midge, PvLEA4, acts as a molecular shield by limiting growth of aggregating protein particles

Hatanaka, R., Hagiwara-Komoda, Y., Furuki, T., Kanamori, Y., Fujitaa, M., Cornette, R., Sakurai, M, Okuda, T. and Kikawada, T.

Insect Biochem. Mol. Biol., ***43(11)****, 1055-1067 (2013)*

LEA proteins are found in anhydrobiotes and are thought to be associated with the acquisition of desiccation tolerance. The sleeping chironomid *Polypedilum vanderplanki*, which can survive in an almost completely desiccated state throughout the larval stage, accumulates LEA proteins in response to desiccation and high salinity conditions. However, the biochemical functions of these proteins remain unclear. Here, we report the characterization of a novel chironomid LEA protein, PvLEA4, which is the most highly accumulated LEA protein in desiccated larvae. Cytoplasmic-soluble PvLEA4 showed many typical characteristics of group 3 LEA proteins (G3LEAs), such as desiccation-inducible accumulation, high hydrophilicity, folding into α-helices on drying, and the ability to reduce aggregation of dehydration-sensitive proteins. This last property of LEA proteins has been termed molecular shield function. To further investigate the molecular shield activity of PvLEA4, we introduced two distinct methods, turbidity measurement and dynamic light scattering (DLS). Turbidity measurements demonstrated that both PvLEA4, and BSA as a positive control, reduced aggregation in α-casein subjected to desiccation and rehydration. However, DLS experiments showed that a small amount of BSA relative to α-casein increased aggregate particle size, whereas PvLEA4 decreased particle size in a dose-dependent manner. Trehalose, which is the main heamolymph sugar in most insects but also a protectant as a chemical chaperone in the sleeping chironomid, has less effect on the limitation of aggregate formation. This analysis suggests that molecular shield proteins function by limiting the growth of protein aggregates during drying and that PvLEA4 counteracts protein aggregation in the desiccation-tolerant larvae of the sleeping chironomid.

3.2061 The Endoplasmic Reticulum Acts as a Platform for Ubiquitylated Components of Nuclear Factor {kappa}B Signaling

Alexia, C., Poales, K., Carvalho, G., Zemirli, N., Dwyer, J., Dubois, S.M., hatchi, E.M., Cordeiro, N., Smith, S.S., Castanier, C., Le Guelte, A., Wan, L., kang, Y., Vazquez, A., Gavard, J., Arnoult, D. and Bidere, N.

Science Signaling, ***6(291)****, ra79 (2013)*

The innate and adaptive immune responses involve the stimulation of nuclear factor κB (NF-κB) transcription factors through the Lys^{63} (K^{63})–linked ubiquitylation of specific components of NF-κB signaling pathways. We found that ubiquitylated components of the NF-κB pathway accumulated on the cytosolic leaflet of the endoplasmic reticulum (ER) membrane after the engagement of cell-surface, proinflammatory cytokine receptors or antigen receptors. Through mass spectrometric analysis, we found that the ER-anchored protein metadherin (MTDH) was a partner for these ubiquitylated activators of NF-κB and that it directly bound to K^{63}-linked polyubiquitin chains. Knockdown of MTDH inhibited the accumulation of ubiquitylated NF-κB signaling components at the ER, reduced the extent of NF-κB activation, and decreased the amount of proinflammatory cytokines produced. Our observations highlight an unexpected facet of the ER as a key subcellular gateway for NF-κB activation.

3.2062 Lypd6 Enhances Wnt/β-Catenin Signaling by Promoting Lrp6 Phosphorylation in Raft Plasma Membrane Domains

Özhan, G., Sezgin, E., Wehner, D., Pfister, A.S., Kühl, S.J., Kagermeier-Schenk, B., Kühl, M., Schwille, P. and Weidinger, G.

*Developmental Cell, **26**, 331-345 (2013)*

Wnt/ β -catenin signaling plays critical roles during embryogenesis, tissue homeostasis, and regeneration. How Wnt-receptor complex activity is regulated is not yet fully understood. Here, we identify the Ly6 family protein LY6/PLAUR domain-containing 6 (Lypd6) as a positive feedback regulator of Wnt/ β -catenin signaling. *lypd6* enhances Wnt signaling in zebrafish and *Xenopus* embryos and in mammalian cells, and it is required for *wnt8*-mediated patterning of the mesoderm and neuroectoderm during zebrafish gastrulation. Lypd6 is GPI anchored to the plasma membrane and physically interacts with the Wnt receptor Frizzled8 and the coreceptor Lrp6. Biophysical and biochemical evidence indicates that Lypd6 preferentially localizes to raft membrane domains, where Lrp6 is phosphorylated upon Wnt stimulation. *lypd6* knockdown or mislocalization of the Lypd6 protein to nonraft membrane domains shifts Lrp6 phosphorylation to these domains and inhibits Wnt signaling. Thus, Lypd6 appears to control Lrp6 activation specifically in membrane rafts, which is essential for downstream signaling.

3.2063 Notch3 is activated by chronic hypoxia and contributes to the progression of human prostate cancer

Danza, G., Di Serio, C., Ambrosio, M.R., Sturli, N., Lonetto, G., Rosati, F., Rocca, B.J., Ventimiglia, G., del Vecchio, M.T., Prudovsky, I., Marchionni, N and Tarantini, F.

*Int. J. Cancer, **133(11)**, 2577-2586 (2013)*

Prostate cancer (PC) is still the second cause of cancer-related death among men. Although patients with metastatic presentation have an ominous outcome, the vast majority of PCs are diagnosed at an early stage. Nonetheless, even among patients with clinically localized disease the outcome may vary considerably. Other than androgen sensitivity, little is known about which other signaling pathways are deranged in aggressive, localized cancers. The elucidation of such pathways may help to develop innovative therapies aimed at specific molecular targets. We report that in a hormone-sensitive PC cell line, LNCaP, Notch3 was activated by hypoxia and sustained cell proliferation and colony formation in soft agar. Hypoxia also modulated cellular cholesterol content and the number and size of lipid rafts, causing a coalescence of small rafts into bigger clusters; under this experimental condition, Notch3 migrated from the non-raft into the raft compartment where it colocalized with the γ-secretase complex. We also looked at human PC biopsies and found that expression of Notch3 positively correlated with Gleason score and with expression of carbonic anhydrase IX, a marker of hypoxia. In conclusion, hypoxia triggers the activation of Notch3, which, in turn, sustains proliferation of PC cells. Notch3 pathway represents a promising target for adjuvant therapy in patients with PC.

3.2064 HIV-1 Nef disrupts membrane-microdomain-associated anterograde transport for plasma membrane delivery of selected Src family kinases

Pan, X., Geist, M.M., Rudolph, J.M., Nickel, W. and fackler, O.T.

*Cell. Microbiol., **15(10)**, 1605-1621 (2013)*

HIV-1 Nef, an essential factor in AIDS pathogenesis, boosts virus replication *in vivo*. As one of its activities in $CD4^+$ T-lymphocytes, Nef potently retargets the Src family kinase (SFK) Lck but not closely related Fyn from the plasma membrane to recycling endosomes and the trans-Golgi network to tailor T-cell activation and optimize virus replication. Investigating the underlying mechanism we find Lck retargeting involves removal of the kinase from membrane microdomains. Moreover, Nef interferes with rapid vesicular transport of Lck to block anterograde transport and plasma membrane delivery of newly synthesized Lck. The sensitivity of Lck to Nef does not depend on functional domains of Lck but requires membrane insertion of the kinase. Surprisingly, the short N-terminal SH4 domain membrane anchor of Lck is necessary and sufficient to confer sensitivity to Nef-mediated anterograde transport block and microdomain extraction. In contrast, the SH4 domain of Fyn is inert to Nef-mediated manipulation. Nef thus interferes with a specialized membrane microdomain-associated pathway for plasma membrane delivery of newly synthesized Lck whose specificity is determined by the affinity of cargo for these sorting platforms. These results provide new insight into the mechanism of Nef action and the pathways used for SFK plasma membrane delivery.

3.2065 The maize methylome influences mRNA splice sites and reveals widespread paramutation-like switches guided by small RNA
Regulski, M., Lu, Z., Kendall, J. et al
Genome Res., ***23***, *1651-1662 (2013)*

The maize genome, with its large complement of transposons and repeats, is a paradigm for the study of epigenetic mechanisms such as paramutation and imprinting. Here, we present the genome-wide map of cytosine methylation for two maize inbred lines, B73 and Mo17. CG (65%) and CHG (50%) methylation (where H = A, C, or T) is highest in transposons, while CHH (5%) methylation is likely guided by 24-nt, but not 21-nt, small interfering RNAs (siRNAs). Correlations with methylation patterns suggest that CG methylation in exons (8%) may deter insertion of *Mutator* transposon insertion, while CHG methylation at splice acceptor sites may inhibit RNA splicing. Using the methylation map as a guide, we used low-coverage sequencing to show that parental methylation differences are inherited by recombinant inbred lines. However, frequent methylation switches, guided by siRNA, persist for up to eight generations, suggesting that epigenetic inheritance resembling paramutation is much more common than previously supposed. The methylation map will provide an invaluable resource for epigenetic studies in maize.

3.2066 Use of an anti-apoptotic CHO cell line for transient gene expression
Macaraeg, N.F., Reilly, D.E. and Womg, A.W.
Biotechnol. Prog., ***29***, *1050-1058 (2013)*

Transient gene expression in mammalian cells allows for rapid production of recombinant proteins for research and preclinical studies. Here, we describe the development of a polyethylenimine (PEI) transient transfection system using an anti-apoptotic host cell line. The host cell line, referred to as the Double Knockout (DKO), was generated by deleting two pro-apoptotic factors, Bax and Bak, in a CHO-K1 cell line using zinc finger nuclease mediated gene disruption. Optimized DNA and PEI volumes for DKO transfections were 50% and 30% lower than CHO-K1, respectively. During transfection DKO cells produced relatively high levels of lactate, but this was mitigated by a temperature shift to 31°C which further enhanced productivity. DKO cells expressed ~3- to 4-fold higher antibody titers than CHO-K1 cells. As evidence of their anti-apoptotic properties post-transfection, DKO cells maintained higher viability and had reduced levels of active caspase-3 compared to CHO-K1 cells. Nuclear plasmid DNA copy numbers and message levels were significantly elevated in DKO cells. Although DNA uptake levels, as early as 40 min post-transfection, were higher in DKO cells this was not due to differences in cell surface heparan sulfate (HS) or initial endocytosis mechanism as both cell types utilized caveolae- and clathrin-mediated endocytosis to internalize DNA:PEI complexes. These results suggest that the increased transfection efficiency and titers from DKO cells are attributed to their resistance to transfection-induced apoptosis and not differences in endocytosis mechanism.

3.2067 LRRK2 secretion in exosomes is regulated by 14-3-3
Fraser, K.B., Moehle, M.S., Daher, J.P.L., Webber, P.J., Williams, J.Y., Stewart, C.A., Yacoubian, T.A., Cowell, r.M., Dokland, T., Ye, t., Chen, D., Siegal, G.P., Galemmo, R.A., Tsika, E., Moore, D.J., Strandaert, D.G., Kojima, K., Mobley, J.A. and West, A.B.
Human. Mol. Genet., ***22(24)***, *4988-5000 (2013)*

Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene cause late-onset Parkinson's disease (PD). Emerging evidence suggests a role for LRRK2 in the endocytic pathway. Here, we show that LRRK2 is released in extracellular microvesicles (i.e. exosomes) from cells that natively express LRRK2. LRRK2 localizes to collecting duct epithelial cells in the kidney that actively secrete exosomes into urine. Purified urinary exosomes contain LRRK2 protein that is both dimerized and phosphorylated. We provide a quantitative proteomic profile of 1673 proteins in urinary exosomes and find that known LRRK2 interactors including 14-3-3 are some of the most abundant exosome proteins. Disruption of the 14-3-3 LRRK2 interaction with a 14-3-3 inhibitor or through acute LRRK2 kinase inhibition potently blocks LRRK2 release in exosomes, but familial mutations in LRRK2 had no effect on secretion. LRRK2 levels were overall comparable but highly variable in urinary exosomes derived from PD cases and age-matched controls, although very high LRRK2 levels were detected in some PD affected cases. We further characterized LRRK2 exosome release in neurons and macrophages in culture, and found that LRRK2-positive exosomes circulate in cerebral spinal fluid (CSF). Together, these results define a pathway for LRRK2 extracellular release, clarify one function of the LRRK2 14-3-3 interaction and provide a foundation for utilization of LRRK2 as a biomarker in clinical trials.

3.2068 Vesicular Transport of Progeny Parvovirus Particle through ER and Golgi Regulates Maturation and Cytolysis
Bär, S., Rommelaere, J. and Nüesch, J.P.F
*PloS Pathogens, **9(9)**, e1003605 (2013)*

Progeny particles of non-enveloped lytic parvoviruses were previously shown to be actively transported to the cell periphery through vesicles in a gelsolin-dependent manner. This process involves rearrangement and destruction of actin filaments, while microtubules become protected throughout the infection. Here the focus is on the intracellular egress pathway, as well as its impact on the properties and release of progeny virions. By colocalization with cellular marker proteins and specific modulation of the pathways through over-expression of variant effector genes transduced by recombinant adeno-associated virus vectors, we show that progeny PV particles become engulfed into COPII-vesicles in the endoplasmic reticulum (ER) and are transported through the Golgi to the plasma membrane. Besides known factors like sar1, sec24, rab1, the ERM family proteins, radixin and moesin play (an) essential role(s) in the formation/loading and targeting of virus-containing COPII-vesicles. These proteins also contribute to the transport through ER and Golgi of the well described analogue of cellular proteins, the secreted Gaussia luciferase in absence of virus infection. It is therefore likely that radixin and moesin also serve for a more general function in cellular exocytosis. Finally, parvovirus egress via ER and Golgi appears to be necessary for virions to gain full infectivity through post-assembly modifications (e.g. phosphorylation). While not being absolutely required for cytolysis and progeny virus release, vesicular transport of parvoviruses through ER and Golgi significantly accelerates these processes pointing to a regulatory role of this transport pathway.

3.2069 10E,12Z-conjugated linoleic acid impairs adipocyte triglyceride storage by enhancing fatty acid oxidation, lipolysis, and mitochondrial reactive oxygen species
Den Hartigh, L.J., Han, C.Y., Wang, S., Omer, M. and Chait, A.
*J. Lipid Res., **54**, 2964-2978 (2013)*

Conjugated linoleic acid (CLA) is a naturally occurring dietary trans fatty acid found in food from ruminant sources. One specific CLA isomer, 10E,12Z-CLA, has been associated with health benefits, such as reduced adiposity, while simultaneously promoting deleterious effects, such as systemic inflammation, insulin resistance, and dyslipidemia. The precise mechanisms by which 10E,12Z-CLA exerts these effects remain unknown. Despite potential health consequences, CLA continues to be advertised as a natural weight loss supplement, warranting further studies on its effects on lipid metabolism. We hypothesized that 10E,12Z-CLA impairs lipid storage in adipose tissue by altering the lipid metabolism of white adipocytes. We demonstrate that 10E,12Z-CLA reduced triglyceride storage due to enhanced fatty acid oxidation and lipolysis, coupled with diminished glucose uptake and utilization in cultured adipocytes. This switch to lipid utilization was accompanied by a potent proinflammatory response, including the generation of cytokines, monocyte chemotactic factors, and mitochondrial superoxide. Disrupting fatty acid oxidation restored glucose utilization and attenuated the inflammatory response to 10E,12Z-CLA, suggesting that fatty acid oxidation is critical in promoting this phenotype. With further investigation into the biochemical pathways involved in adipocyte responses to 10E,12Z-CLA, we can discern more information about its safety and efficacy in promoting weight loss.

3.2070 Novel Staphylococcal Glycosyltransferases SdgA and SdgB Mediate Immunogenicity and Protection of Virulence-Associated Cell Wall Proteins
Hazenbos, W.L. et al
*PloS Pathogens, **9(10)**, e1003653 (2013)*

Infection of host tissues by *Staphylococcus aureus* and *S. epidermidis* requires an unusual family of staphylococcal adhesive proteins that contain long stretches of serine-aspartate dipeptide-repeats (SDR). The prototype member of this family is clumping factor A (ClfA), a key virulence factor that mediates adhesion to host tissues by binding to extracellular matrix proteins such as fibrinogen. However, the biological siginificance of the SDR-domain and its implication for pathogenesis remain poorly understood. Here, we identified two novel bacterial glycosyltransferases, SdgA and SdgB, which modify all SDR-proteins in these two bacterial species. Genetic and biochemical data demonstrated that these two glycosyltransferases directly bind and covalently link N-acetylglucosamine (GlcNAc) moieties to the SDR-domain in a step-wise manner, with SdgB appending the sugar residues proximal to the target Ser-Asp repeats, followed by additional modification by SdgA. GlcNAc-modification of SDR-proteins by SdgB creates an immunodominant epitope for highly opsonic human antibodies, which represent up to 1% of total human IgG. Deletion of these glycosyltransferases renders SDR-proteins vulnerable to proteolysis

by human neutrophil-derived cathepsin G. Thus, SdgA and SdgB glycosylate staphylococcal SDR-proteins, which protects them against host proteolytic activity, and yet generates major eptopes for the human anti-staphylococcal antibody response, which may represent an ongoing competition between host and pathogen.

3.2071 Combined enrichment of neuromelanin granules and synaptosomes from human substantia nigra pars compacta tissue for proteomic analysis

Plum, A., Helling, S., Theiss, C., Leite, R.E.P., May, C., Jacob-Filho, W., Eisenacher, M., Kuhlmann, K., Meyer, H.E., Riederer, P., Grinberg, L.T., Gerlach, M. and Marcus, K.
*J. Proteomics, **94**, 202-206 (2013)*

This article gives a detailed description of a protocol using density gradient centrifugation for the enrichment of neuromelanin granules and synaptosomes from low amounts (≥ 0.15 g) of human *substantia nigra pars compacta* tissue. This has a great advantage compared to already existing methods as it allows for the first time (i) a combined enrichment of neuromelanin granules and synaptosomes and (ii) just minimal amounts of tissue necessary to enable donor specific analysis. Individual specimens were classified as control or diseased according to clinical evaluation and neuropathological examination. For the enrichment of synaptosomes and neuromelanin granules from the same tissue sample density gradient centrifugations using Percoll® and Iodixanol were performed. The purity of resulting fractions was checked by transmission electron microscopy. We were able to establish a reproducible and easy to handle protocol combining two different density gradient centrifugations: using an Iodixanol gradient neuromelanin granules were enriched and in parallel, from the same sample, a fraction of synaptosomes with high purity using a Percoll® gradient was obtained. Our subfractionation strategy will enable a subsequent in depth proteomic characterization of neurodegenerative processes in the *substantia nigra pars compacta* in patients with Parkinson's disease and dementia with Lewy bodies compared to appropriate controls.

3.2072 Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles

Kooijimans, S.A.A., Streemersch, S., Braeckmans, K., de Smedt, S.C., Hendrix, A., Wood, M.J.A., Schiffelers, R.M., Raemdonck, K. and Vader, P.
J. Controlled Release, 172, 229-238 (2013)

Extracellular vesicles (EVs) are specialised endogenous carriers of proteins and nucleic acids and are involved in intercellular communication. EVs are therefore proposed as candidate drug delivery systems for the delivery of nucleic acids and other macromolecules. However, the preparation of EV-based drug delivery systems is hampered by the lack of techniques to load the vesicles with nucleic acids. In this work we have now characterised in detail the use of an electroporation method for this purpose. When EVs were electroporated with fluorescently labelled siRNA, siRNA retention was comparable with previously published results (20–25% based on fluorescence spectroscopy and fluorescence fluctuation spectroscopy), and electroporation with unlabelled siRNA resulted in significant siRNA retention in the EV pellet as measured by RT-PCR. Remarkably, when siRNA was electroporated in the absence of EVs, a similar or even greater siRNA retention was measured. Nanoparticle tracking analysis and confocal microscopy showed extensive formation of insoluble siRNA aggregates after electroporation, which could be dramatically reduced by addition of EDTA. Other strategies to reduce aggregate formation, including the use of cuvettes with conductive polymer electrodes and the use of an acidic citrate electroporation buffer, resulted in a more efficient reduction of siRNA precipitation than EDTA. However, under these conditions, siRNA retention was below 0.05% and no significant differences in siRNA retention could be measured between samples electroporated in the presence or absence of EVs. Our results show that electroporation of EVs with siRNA is accompanied by extensive siRNA aggregate formation, which may cause overestimation of the amount of siRNA actually loaded into EVs. Moreover, our data clearly illustrate that electroporation is far less efficient than previously described, and highlight the necessity for alternative methods to prepare siRNA-loaded EVs.

3.2073 Protection of a Ceramide Synthase 2 Null Mouse from Drug-induced Liver Injury: ROLE OF GAP JUNCTION DYSFUNCTION AND CONNEXIN 32 MISLOCALIZATION

Park, W-J., Park, J-W., Erez-Roman, R., Kogot-Levin, A., Bame, J.R., Tirosh, B., Saada, A., Merrill Jr., A.H., Pewzner-Jung, Y. and Futerman, H.
*J. Biol. Chem., **288(43)**, 309004-30916 (2013)*

Very long chain (C22-C24) ceramides are synthesized by ceramide synthase 2 (CerS2). A CerS2 null mouse displays hepatopathy because of depletion of C22-C24 ceramides, elevation of C16-ceramide, and/or elevation of sphinganine. Unexpectedly, CerS2 null mice were resistant to acetaminophen-induced hepatotoxicity. Although there were a number of biochemical changes in the liver, such as increased levels of glutathione and multiple drug-resistant protein 4, these effects are unlikely to account for the lack of acetaminophen toxicity. A number of other hepatotoxic agents, such as d-galactosamine, CCl_4, and thioacetamide, were also ineffective in inducing liver damage. All of these drugs and chemicals require connexin (Cx) 32, a key gap junction protein, to induce hepatotoxicity. Cx32 was mislocalized to an intracellular location in hepatocytes from CerS2 null mice, which resulted in accelerated rates of its lysosomal degradation. This mislocalization resulted from the altered membrane properties of the CerS2 null mice, which was exemplified by the disruption of detergent-resistant membranes. The lack of acetaminophen toxicity and Cx32 mislocalization were reversed upon infection with recombinant adeno-associated virus expressing CerS2. We establish that Gap junction function is compromised upon altering the sphingolipid acyl chain length composition, which is of relevance for understanding the regulation of drug-induced liver injury.

3.2074 **Interaction Maps of the Saccharomyces cerevisiae ESCRT-III Protein Snf7**
Sciskala, B. and Kölling, R.
*Eukaryot. Cell, **12(11)**, 1538-1546 (2013)*

The Saccharomyces cerevisiae ESCRT-III protein Snf7 is part of an intricate interaction network at the endosomal membrane. Interaction maps of Snf7 were established by measuring the degree of binding of individual binding partners to putative binding motifs along the Snf7 sequence by glutathione *S*-transferase (GST) pulldown. For each interaction partner, distinct binding profiles were obtained. The following observations were made. The ESCRT-III subunits Vps20 and Vps24 showed a complementary binding pattern, suggesting a model for the series of events in the ESCRT-III functional cycle. Vps4 bound to individual Snf7 motifs but not to full-length Snf7. This suggests that Vps4 does not bind to the closed conformation of Snf7. We also demonstrate for the first time that the ALIX/Bro1 homologue Rim20 binds to the α6 helix of Snf7. Analysis of a Snf7 α6 deletion mutant showed that the α6 helix is crucial for binding of Bro1 and Rim20 *in vivo* and is indispensable for the multivesicular body (MVB)-sorting and Rim-signaling functions of Snf7. The Snf7Δα6 protein still appeared to be incorporated into ESCRT-III complexes at the endosomal membrane, but disassembly of the complex seemed to be defective. In summary, our study argues against the view that the ESCRT cycle is governed by single one-to-one interactions between individual components and emphasizes the network character of the ESCRT interactions.

3.2075 **Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia**
Baginska, J., Viry, E., Berchem, G., Poli, A., Noman, Z., van Moer, K., Medves, S., Zimmer, J., Oudin, A., Niclou, S.P., Bleackley, R.C., Goping, I.S., Chouaib, S. and Janji, B.
*PNAS, **110(43)**, 17450-17455 (2013)*

Recent studies demonstrated that autophagy is an important regulator of innate immune response. However, the mechanism by which autophagy regulates natural killer (NK) cell-mediated antitumor immune responses remains elusive. Here, we demonstrate that hypoxia impairs breast cancer cell susceptibility to NK-mediated lysis in vitro via the activation of autophagy. This impairment was not related to a defect in target cell recognition by NK cells but to the degradation of NK-derived granzyme B in autophagosomes of hypoxic cells. Inhibition of autophagy by targeting beclin1 (BECN1) restored granzyme B levels in hypoxic cells in vitro and induced tumor regression in vivo by facilitating NK-mediated tumor cell killing. Together, our data highlight autophagy as a mechanism underlying the resistance of hypoxic tumor cells to NK-mediated lysis. The work presented here provides a cutting-edge advance in our understanding of the mechanism by which hypoxia-induced autophagy impairs NK-mediated lysis in vitro and paves the way for the formulation of more effective NK cell-based antitumor therapies.

3.2076 **Pooled RNAi screen identifies ubiquitin ligase Itch as crucial for influenza A virus release from the endosome during virus entry**
Su, W-S., Chen, Y-C., Tseng, C-H., Hsu, P.W-C., Tung, K-F., jeng, K-S. and Lai, M.M.C.
*PNAS, **110(43)**, 17516-17521 (2013)*

Influenza viruses, like other viruses, rely on host factors to support their life cycle as viral proteins usually "hijack," or collaborate with, cellular proteins to execute their functions. Identification and understanding of these factors can increase the knowledge of molecular mechanisms manipulated by the viruses and facilitate development of antiviral drugs. To this end, we developed a unique genome-wide pooled shRNA screen to search for cellular factors important for influenza A virus (IAV) replication. We identified an E3 ubiquitin ligase, Itch, as an essential factor for an early step in the viral life cycle. In Itch knockdown cells, the incorporation of viral ribonucleoprotein complex into endosomes was normal, but its subsequent release from endosomes and transport to the nucleus was retarded. In addition, upon virus infection, Itch was phosphorylated and recruited to the endosomes, where virus particles were located. Furthermore, Itch interacted with viral M1 protein and ubiquitinated M1 protein. Collectively, our findings unravel a critical role of Itch in mediating IAV release from the endosome and offer insights into the mechanism for IAV uncoating during virus entry. These findings also highlight the feasibility of pooled RNAi screening for exploring the cellular cofactors of lytic viruses.

3.2077 **A tuberous sclerosis complex signalling node at the peroxisome regulates mTORC1 and autophagy in response to ROS**
Zhang, J. et al
Nature Cell Biol., ***15(10)***, *1186-1196 (2013)*

Subcellular localization is emerging as an important mechanism for mTORC1 regulation. We report that the tuberous sclerosis complex (TSC) signalling node, TSC1, TSC2 and Rheb, localizes to peroxisomes, where it regulates mTORC1 in response to reactive oxygen species (ROS). TSC1 and TSC2 were bound by peroxisomal biogenesis factors 19 and 5 (PEX19 and PEX5), respectively, and peroxisome-localized TSC functioned as a Rheb GTPase-activating protein (GAP) to suppress mTORC1 and induce autophagy. Naturally occurring pathogenic mutations in TSC2 decreased PEX5 binding, and abrogated peroxisome localization, Rheb GAP activity and suppression of mTORC1 by ROS. Cells lacking peroxisomes were deficient in mTORC1 repression by ROS, and peroxisome-localization-deficient TSC2 mutants caused polarity defects and formation of multiple axons in neurons. These data identify a role for the TSC in responding to ROS at the peroxisome, and identify the peroxisome as a signalling organelle involved in regulation of mTORC1.

3.2078 **Functionally Diverse MicroRNA Effector Complexes Are Regulated by Extracellular Signaling**
Wu, P-H., Isaji, M. and carthew, R.W.
Molecular Cell, ***52(1)***, *113-123 (2013)*

Because microRNAs (miRNAs) influence the expression of many genes in cells, discovering how the miRNA pathway is regulated is an important area of investigation. We found that the *Drosophila* miRNA-induced silencing complex (miRISC) exists in multiple forms. A constitutive form, called G-miRISC, is comprised of Ago1, miRNA, and GW182. Two distinct miRISC complexes that lack GW182 are regulated by mitogenic signaling. Exposure of cells to serum, lipids, or the tumor promoter PMA suppressed formation of these complexes. P-miRISC is comprised of Ago1, miRNA, and Loqs-PB, and it associates with mRNAs assembled into polysomes. The other regulated Ago1 complex associates with membranous organelles and is likely an intermediate in miRISC recycling. The formation of these complexes is correlated with a 5- to 10-fold stronger repression of target gene expression inside cells. Taken together, these results indicate that mitogenic signaling regulates the miRNA effector machinery to attenuate its repressive activities.

3.2079 **Plant Sterols the Better Cholesterol in Alzheimer's Disease? A Mechanistical Study**
Burg, V.K. et al
J. Neurosci., ***33(41)***, *16072-16087 (2013)*

Amyloid-β (Aβ), major constituent of senile plaques in Alzheimer's disease (AD), is generated by proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretase. Several lipids, especially cholesterol, are associated with AD. Phytosterols are naturally occurring cholesterol plant equivalents, recently been shown to cross the blood–brain-barrier accumulating in brain. Here, we investigated the effect of the most nutritional prevalent phytosterols and cholesterol on APP processing. In general, phytosterols are less amyloidogenic than cholesterol. However, only one phytosterol, stigmasterol, reduced Aβ generation by (1) directly decreasing β-secretase activity, (2) reducing expression of all γ-secretase components, (3) reducing cholesterol and presenilin distribution in lipid rafts implicated in amyloidogenic APP cleavage, and by (4) decreasing BACE1 internalization to endosomal compartments,

involved in APP β-secretase cleavage. Mice fed with stigmasterol-enriched diets confirmed protective effects *in vivo*, suggesting that dietary intake of phytosterol blends mainly containing stigmasterol might be beneficial in preventing AD.

3.2080 Oncogenic K-ras segregates at spatially distinct plasma membrane signaling platforms according to its phosphorylation status

Barcelo, C., Paco, N., Beckett, A.J., Alvarez-Moya, B., Garrido, E., Gelabert, M., Tebar, F., Jaumot, M., Prior, I. and Agell, N.
*J. Cell. Science, **126(20)**, 4553-4559 (2013)*

Activating mutations in the K-Ras small GTPase are extensively found in human tumors. Although these mutations induce the generation of a constitutively GTP-loaded, active form of K-Ras, phosphorylation at Ser181 within the C-terminal hypervariable region can modulate oncogenic K-Ras function without affecting the *in vitro* affinity for its effector Raf-1. In striking contrast, K-Ras phosphorylated at Ser181 shows increased interaction in cells with the active form of Raf-1 and with p110α, the catalytic subunit of PI 3-kinase. Because the majority of phosphorylated K-Ras is located at the plasma membrane, different localization within this membrane according to the phosphorylation status was explored. Density-gradient fractionation of the plasma membrane in the absence of detergents showed segregation of K-Ras mutants that carry a phosphomimetic or unphosphorylatable serine residue (S181D or S181A, respectively). Moreover, statistical analysis of immunoelectron microscopy showed that both phosphorylation mutants form distinct nanoclusters that do not overlap. Finally, induction of oncogenic K-Ras phosphorylation – by activation of protein kinase C (PKC) – increased its co-clustering with the phosphomimetic K-Ras mutant, whereas (when PKC is inhibited) non-phosphorylated oncogenic K-Ras clusters with the non-phosphorylatable K-Ras mutant. Most interestingly, PI 3-kinase (p110α) was found in phosphorylated K-Ras nanoclusters but not in non-phosphorylated K-Ras nanoclusters. In conclusion, our data provide – for the first time – evidence that PKC-dependent phosphorylation of oncogenic K-Ras induced its segregation in spatially distinct nanoclusters at the plasma membrane that, in turn, favor activation of Raf-1 and PI 3-kinase.

3.2081 Lipid droplet breakdown requires Dynamin 2 for vesiculation of autolysosomal tubules in hepatocytes

Schulze, R.J., Weller, S.G., Schroeder, B., Krueger, E.W., Chi, S., Casey, C.A. and McNiven, M.A.
*J. Cell Biol., **203(2)**, 315-326 (2013)*

Lipid droplets (LDs) are lipid storage organelles that in hepatocytes may be catabolized by autophagy for use as an energy source, but the membrane-trafficking machinery regulating such a process is poorly characterized. We hypothesized that the large GTPase Dynamin 2 (Dyn2), well known for its involvement in membrane deformation and cellular protein trafficking, could orchestrate autophagy-mediated LD breakdown. Accordingly, depletion or pharmacologic inhibition of Dyn2 led to a substantial accumulation of LDs in hepatocytes. Strikingly, the targeted disruption of Dyn2 induced a dramatic four- to fivefold increase in the size of autolysosomes. Chronic or acute Dyn2 inhibition combined with nutrient deprivation stimulated the excessive tubulation of these autolysosomal compartments. Importantly, Dyn2 associated with these tubules along their length, and the tubules vesiculated and fragmented in the presence of functional Dyn2. These findings provide new evidence for the participation of the autolysosome in LD metabolism and demonstrate a novel role for dynamin in the function and maturation of an autophagic compartment.

3.2082 Polar substitutions in helix 3 of the prion protein produce transmembrane isoforms that disturb vesicle trafficking

Sanchez-garcia, J., Arbelaez, D., Jensen, K., Rincon-Limas, D.E. and Fernandez-Funez, P.
*Hum. Mol. Genet., **22(21)**, 4253-4266 (2013)*

Prion diseases encompass a diverse group of neurodegenerative conditions characterized by the accumulation of misfolded prion protein (PrP) isoforms. Other conformational variants of PrP have also been proposed to contribute to neurotoxicity in prion diseases, including misfolded intermediates as well as cytosolic and transmembrane isoforms. To better understand PrP neurotoxicity, we analyzed the role of two highly conserved methionines in helix 3 on PrP biogenesis, folding and pathogenesis. Expression of the PrP-M205S and -M205,212S mutants in *Drosophila* led to hyperglycosylation, intracellular accumulation and widespread conformational changes due to failure of oxidative folding. Surprisingly, PrP-M205S and -M205,212S acquired a transmembrane topology (Ctm) previously linked to mutations in

the signal peptide (SP) and the transmembrane domain (TMD). PrP-M205,212S also disrupted the accumulation of key neurodevelopmental proteins in lipid rafts, resulting in shortened axonal projections. These results uncover a new role for the hydrophobic domain in promoting oxidative folding and preventing the formation of neurotoxic Ctm PrP, mechanisms that may be relevant in the pathogenesis of both inherited and sporadic prion diseases.

3.2083 Wnt5a Directs Polarized Calcium Gradients by Recruiting Cortical Endoplasmic Reticulum to the Cell Trailing Edge

Witze, E.S., Connacher, K.C., Houel, S., Schwartz, M.P., Morphew, M.K., Reid, L., Sacks, D.B., Anseth, K.S. and Ahn, N.G.

*Developmental Cell, **26(6)**, 645-657 (2013)*

Wnt5a directs the assembly of the Wnt-receptor-actin-myosin-polarity (WRAMP) structure, which integrates cell-adhesion receptors with F-actin and myosin to form a microfilament array associated with multivesicular bodies (MVBs). The WRAMP structure is polarized to the cell posterior, where it directs tail-end membrane retraction, driving forward translocation of the cell body. Here we define constituents of the WRAMP proteome, including regulators of microfilament and microtubule dynamics, protein interactions, and enzymatic activity. IQGAP1, a scaffold for F-actin nucleation and crosslinking, is necessary for WRAMP structure formation, potentially bridging microfilaments and MVBs. Vesicle coat proteins, including coatomer-I subunits, localize to and are required for the WRAMP structure. Electron microscopy and live imaging demonstrate movement of the ER to the WRAMP structure and plasma membrane, followed by elevation of intracellular Ca^{2+}. Thus, Wnt5a controls directional movement by recruiting cortical ER to mobilize a rear-directed, localized Ca^{2+} signal, activating actomyosin contraction and adhesion disassembly for membrane retraction.

3.2084 The biogenesis protein PEX14 is an optimal marker for the identification and localization of peroxisomes in different cell types, tissues, and species in morphological studies

Grant, P., ahlemeyer, B., Karnati, S., Berg, T., Stelzig, I., Nenicu, A., Kuchelmeister, K., Crane, D.I. and Baumgart-Vogt, E.

*Histochem. Cell. Biol., **140(4)**, 423-442 (2013)*

Catalase and ABCD3 are frequently used as markers for the localization of peroxisomes in morphological experiments. Their abundance, however, is highly dependent on metabolic demands, reducing the validity of analyses of peroxisomal abundance and distribution based solely on these proteins. We therefore attempted to find a protein which can be used as an optimal marker for peroxisomes in a variety of species, tissues, cell types and also experimental designs, independently of peroxisomal metabolism. We found that the biogenesis protein peroxin 14 (PEX14) is present in comparable amounts in the membranes of every peroxisome and is optimally suited for immunoblotting, immunohistochemistry, immunofluorescence, and immunoelectron microscopy. Using antibodies against PEX14, we could visualize peroxisomes with almost undetectable catalase content in various mammalian tissue sections (submandibular and adrenal gland, kidney, testis, ovary, brain, and pancreas from mouse, cat, baboon, and human) and cell cultures (primary cells and cell lines). Peroxisome labeling with catalase often showed a similar tissue distribution to the mitochondrial enzyme mitochondrial superoxide dismutase (both responsible for the degradation of reactive oxygen species), whereas ABCD3 exhibited a distinct labeling only in cells involved in lipid metabolism. We increased the sensitivity of our methods by using QuantumDots™, which have higher emission yields compared to classic fluorochromes and are unsusceptible to photobleaching, thereby allowing more exact quantification without artificial mistakes due to heterogeneity of individual peroxisomes. We conclude that PEX14 is indeed the best marker for labeling of peroxisomes in a variety of tissues and cell types in a consistent fashion for comparative morphometry.

3.2085 Historical Overview of Autophagy

Dunn Jr., W.A., Schroder, L.A. and Aris, J.P.

*Current Cancer Res., **8**, 1-24 (2013)*

This chapter highlights those scientists who founded the field of autophagy (APG) research during its beginnings to those that have made key discoveries to advance the field into the mainstream of science. In the beginning, researchers were interested in lysosome morphology and function and how it related to protein turnover. These early studies were limited to morphological and biochemical methods that were restricted to mammalian cells and organs. APG was thought to be a highly regulated nonselective degradative process that could lead to cell death. When APG was characterized in yeast, a genetic model

emerged allowing the identification of APG-related genes. Soon, new protein markers became available to better monitor and characterize APG in yeast, plants, insects, and animals. We now appreciate that APG has a positive role in cellular homeostasis and cell survival by recycling needed nutrients to sustain cellular functions and removing dysfunctional organelles and intracellular pathogens.

3.2086 Suppression of amyloid-β production by 24S-hydroxycholesterol
Urano, Y., Ochiai, S. and Noguchi, N.
*FASEB J., **27**, 4305-4315 (2013)*

Cholesterol can be converted to 24S-hydroxycholesterol (24SOHC) by neuronal cholesterol 24-hydroxylase. In mouse models of Alzheimer's disease (AD), increasing 24SOHC levels reduced AD pathology. However, mechanisms underlying the effects of 24SOHC on amyloid-β (Aβ) production have remained unclear. Here we report that 24SOHC treatment reduces Aβ production and increases endoplasmic reticulum (ER)-resident immature amyloid precursor protein (APP) levels in human neuroblastoma SH-SY5Y cells and CHO cells stably expressing human APP. Treatment with 1–10 μM 24SOHC (equivalent to the concentrations detected in human brain homogenates) diminished Aβ production (IC_{50}=4.6 μM for $Aβ_{40}$) without affecting secretase activities. To evaluate the intracellular APP transport, we established an *in vitro* vesicle formation assay. We found that APP budding *via* COPII vesicles was diminished by 70% in 24SOHC-treated cells. The proteomics and immunoblotting analysis revealed that 24SOHC induced the expression of glucose-regulated protein 78 (GRP78), an ER chaperone, through unfolded protein response pathways, and enhanced the formation of the APP/GRP78 complex. Knockdown of GRP78 diminished the inhibitory effects of 24SOHC on Aβ production. These results suggest that 24SOHC down-regulates APP trafficking *via* enhancement of the complex formation of APP with up-regulated GRP78 in the ER, resulting in suppression of Aβ production.—Urano, Y., Ochiai, S., Noguchi, N. Suppression of amyloid-β production by 24S-hydroxycholesterol*via* inhibition of intracellular amyloid precursor protein trafficking.

3.2087 LMBD1 Protein Serves as a Specific Adaptor for Insulin Receptor Internalization
Tseng, L.T-L., Lin, C-L., Tzen, K-Y., Chang, S.C. and chang, M-F.
*J. Biol. Chem., **288(45)**, 32424-32432 (2013)*

Energy homeostasis is crucial for maintaining normally functioning cells; disturbances in this balance often cause various diseases. The limb region 1 (LMBR1) domain containing 1 gene (*lmbrd1*) encodes the LMBD1 protein that possesses 9 putative transmembrane domains. LMBD1 has been suggested to be involved in the lysosome in aiding the export of cobalamin. In this study, we determined that LMBD1 plays a regulatory role in the plasma membrane. A micro-positron emission tomography analysis showed that a single-allele knock-out of *lmbrd1* increased the ^{18}F-fluorodeoxyglucose uptake in murine hearts. In addition, the knockdown of *lmbrd1* resulted in an up-regulated signaling of the insulin receptor (IR) and its downstream signaling molecule, Akt. Confocal and live total internal reflection fluorescence microscopy showed that LMBD1 co-localized and co-internalized with clathrin and the IR, but not with the transferrin receptor. The results of the mutation analysis and phenotypic rescue experiments indicate that LMBD1 interacts with adaptor protein-2 and is involved in the unique clathrin-mediated endocytosis of the IR. LMBD1 selectively interacts with the IR. The knockdown of *lmbrd1* attenuated IR endocytosis, resulting in the perturbation of the IR recycling pathway and consequential enhancement of the IR signaling cascade. In summary, LMBD1 plays an imperative role in mediating and regulating the endocytosis of the IR.

3.2088 Pseudomonas aeruginosa Outer Membrane Vesicles Modulate Host Immune Responses by Targeting the Toll-Like Receptor 4 Signaling Pathway
Zhao, K., Deng, X., He, C., Yue, B. and Wu, M.
*Infect. Immun., **83(12)**, 4509-4518 (2013)*

Bacteria can naturally secrete outer membrane vesicles (OMVs) as pathogenic factors, while these vesicles may also serve as immunologic regulators if appropriately prepared. However, it is largely unknown whether Pseudomonas aeruginosa OMVs can activate inflammatory responses and whether immunization with OMVs can provide immune protection against subsequent infection. We purified and identified OMVs, which were then used to infect lung epithelial cells *in vitro* as well as C57BL/6J mice to investigate the immune response and the underlying signaling pathway. The results showed that OMVs generated from P. aeruginosa wild-type strain PAO1 were more cytotoxic to alveolar epithelial cells than those from quorum-sensing (QS)-deficient strain PAO1-Δ*lasR*. The levels of Toll-like receptor 4 (TLR4) and proinflammatory cytokines, including interleukin-1β (IL-1β) and IL-6, increased following OMV

infection. Compared with lipopolysaccharide (LPS), lysed OMVs in which the membrane structures were broken induced a weak immune response. Furthermore, expression levels of TLR4-mediated responders (i.e., cytokines) were markedly downregulated by the TLR4 inhibitor E5564. Active immunization with OMVs or passive transfer of sera with a high cytokine quantity acquired from OMV-immunized mice could protect healthy mice against subsequent lethal PAO1 challenges (1.5×10^{11} CFU). Collectively, these findings indicate that naturally secreted P. aeruginosa OMVs may trigger significant inflammatory responses via the TLR4 signaling pathway and protect mice against pseudomonal lung infection.

3.2089 **Proteomic techniques for characterisation of mesenchymal stem cell secretome**
Skalnikova, H.K.
*Biochimie, **95**, 2196-2211 (2013)*

Mesenchymal stem cells (MSCs) are multipotent cells with a substantial potential in human regenerative medicine due to their ability to migrate to sites of injury, capability to suppress immune response and accessibility in large amount from patient's own bone marrow or fat tissue. It has been increasingly observed that the transplanted MSCs did not necessarily engraft and differentiate at the site of injury but might exert their therapeutic effects through secreted trophic signals. The MSCs secrete a variety of autocrine/paracrine factors, called secretome, that support regenerative processes in the damaged tissue, induce angiogenesis, protect cells from apoptotic cell death and modulate immune system. The cell culture medium conditioned by MSCs or osteogenic, chondrogenic as well as adipogenic precursors derived from MSCs has become a subject of intensive proteomic profiling in the search for and identification of released factors and microvesicles that might be applicable in regenerative medicine. Jointly with the methods for MSC isolation, expansion and differentiation, proteomic analysis of MSC secretome was enabled recently mainly due to the extensive development in protein separation techniques, mass spectrometry, immunological methods and bioinformatics. This review describes proteomic techniques currently applied or prospectively applicable in MSC secretomics, with a particular focus on preparation of the secretome sample, protein/peptide separation, mass spectrometry and protein quantification techniques, analysis of posttranslational modifications, immunological techniques, isolation and characterisation of secreted vesicles and exosomes, analysis of cytokine-encoding mRNAs and bioinformatics.

3.2090 **Lipid raft-regulated IGF-1R activation antagonizes TRAIL-induced apoptosis in gastric cancer cells**
Xu, L., Qu, X., Hu, X., Zhu, Z., Li, C., Li, E., Ma, Y., Song, N. and Liu, Y.
*FEBS Lett., **587**, 3815-3823 (2013)*

Gastric cancer cells are resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the resistance mechanism is not fully understood. In human gastric cancer MGC803 and BGC823 cells, TRAIL induces insulin-like growth factor-1 receptor (IGF-1R) pathway activation. Treatment with IGF-1R inhibitor OSI-906 or small interfering RNAs against IGF-1R, prevents IGF-1R pathway activation and increases TRAIL-induced apoptosis. The TRAIL-induced IGF-1R pathway activation is promoted by IGF-1R translocation into lipid rafts. Moreover, the translocation of IGF-1R into lipid rafts is regulated by Casitas B-lineage lymphoma b (Cbl-b). Taken together, TRAIL-induced IGF-1R activation antagonizes TRAIL-induced apoptosis by Cbl-b-regulated distribution of IGF-1R in lipid rafts.

3.2091 Ctr2 regulates biogenesis of a cleaved form of mammalian Ctr1 metal transporter lacking the copper- and cisplatin-binding ecto-domain
Ohrvik, H., Nose, Y., Wood, L.K., Kim, B-e., Gleber, S-C., Ralle, M. and Thiele, D.J.
PNAS, 110(46), E4279-E4288 (2013)

Copper is an essential catalytic cofactor for enzymatic activities that drive a range of metabolic biochemistry including mitochondrial electron transport, iron mobilization, and peptide hormone maturation. Copper dysregulation is associated with fatal infantile disease, liver, and cardiac dysfunction, neuropathy, and anemia. Here we report that mammals regulate systemic copper acquisition and intracellular mobilization via cleavage of the copper-binding ecto-domain of the copper transporter 1 (Ctr1). Although full-length Ctr1 is critical to drive efficient copper import across the plasma membrane, cleavage of the ecto-domain is required for Ctr1 to mobilize endosomal copper stores. The biogenesis of the truncated form of Ctr1 requires the structurally related, previously enigmatic copper transporter 2 (Ctr2). Ctr2−/− mice are defective in accumulation of truncated Ctr1 and exhibit increased tissue copper levels, and X-ray fluorescence microscopy demonstrates that copper accumulates as intracellular foci. These studies identify a key regulatory mechanism for mammalian copper transport through Ctr2-dependent accumulation of a Ctr1 variant lacking the copper- and cisplatin-binding ecto-domain.

3.2092 Adrenergic Regulation of IgE Involves Modulation of CD23 and ADAM10 Expression on Exosomes
Padro, C.J., Shawler, T.M., Gormley, M.G. and Sanders, V.M:
J. Immunol., 191, 5383-5397 (2013)

Soluble CD23 plays a role in the positive regulation of an IgE response. Engagement of the β2 adrenergic receptor (β2AR) on a B cell is known to enhance the level of both soluble CD23 and IgE, although the mechanism by which this occurs is not completely understood. In this study, we report that, in comparison with a CD40 ligand/IL-4–primed murine B cell alone, β2AR engagement on a primed B cell increased gene expression of a disintegrin and metalloproteinase (ADAM)10, which is the primary sheddase of CD23, as well as protein expression of both CD23 and ADAM10, in a protein kinase A– and p38 MAPK–dependent manner, and promoted the localization of these proteins to exosomes as early as 2 d after priming, as determined by both Western blot and flow cytometry and confirmed by electron microscopy. In comparison with isolated exosomes released from primed B cells alone, the transfer of exosomes released from β2AR agonist–exposed primed B cells to cultures of recipient primed B cells resulted in an increase in the level of IgE produced per cell, without affecting the number of cells producing IgE, as determined by ELISPOT. These effects still occurred when a β2AR antagonist was added along with the transfer to block residual agonist, and they failed to occur when exosomes were isolated from β2AR-deficient B cells. These findings suggest that the mechanism responsible for mediating the β2AR-induced increase in IgE involves a shuttling of the β2AR-induced increase in CD23 and ADAM10 proteins to exosomes that subsequently mediate an increase in IgE.

3.2093 The Adaptor Protein-1 μ1B Subunit Expands the Repertoire of Basolateral Sorting Signal Recognition in Epithelial Cells
Guo, X., Mattera, R., Ren, X., Chen, Y., Retamal, C., Gonzalez, A. and Bonifacino, J.S.
Developmental Cell, 27(3), 353-366 (2013)

An outstanding question in protein sorting is why polarized epithelial cells express two isoforms of the 1 subunit of the AP-1 clathrin adaptor complex: the ubiquitous 1A and the epithelial-specific 1B. Previous studies led to the notion that 1A and 1B mediate basolateral sorting predominantly from the trans-Golgi network (TGN) and recycling endosomes, respectively. Using improved analytical tools, however, we find that 1A and 1B largely colocalize with each other. They also colocalize to similar extents with TGN and recycling endosome markers, as well as with basolateral cargoes transiting biosynthetic and endocytic-recycling routes. Instead, the two isoforms differ in their signal-recognition specificity. In particular, 1B preferentially binds a subset of signals from cargoes that are sorted basolaterally in a 1B-dependent manner. We conclude that expression of distinct 1 isoforms in epithelial cells expands the repertoire of signals recognized by AP-1 for sorting of a broader range of cargoes to the basolateral surface.

3.2094 Syntaxin 16 is a master recruitment factor for cytokinesis
Neto, H., Kaupisch, A., Collins, L.L. and Gould, G.W.
*Mol. Biol. Cell, **24**, 3663-3674 (2013)*

Recently it was shown that both recycling endosome and endosomal sorting complex required for transport (ESCRT) components are required for cytokinesis, in which they are believed to act in a sequential manner to bring about secondary ingression and abscission, respectively. However, it is not clear how either of these complexes is targeted to the midbody and whether their delivery is coordinated. The trafficking of membrane vesicles between different intracellular organelles involves the formation of soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complexes. Although membrane traffic is known to play an important role in cytokinesis, the contribution and identity of intracellular SNAREs to cytokinesis remain unclear. Here we demonstrate that syntaxin 16 is a key regulator of cytokinesis, as it is required for recruitment of both recycling endosome–associated Exocyst and ESCRT machinery during late telophase, and therefore that these two distinct facets of cytokinesis are inextricably linked.

3.2095 Arf-like GTPase Arl8b regulates lytic granule polarization and natural killer cell–mediated cytotoxicity

Tuli, A., Thiery, J., James, A.M., Michelet, X., Sharma, M., Garg, S., Sanborn, K.B., Orange, J.S., Liberman, J. and Brenner, M.B.
Mol. Biol. Cell, ***24***, *3721-3735 (2013)*

Natural killer (NK) lymphocytes contain lysosome-related organelles (LROs), known as lytic granules, which upon formation of immune synapse with the target cell, polarize toward the immune synapse to deliver their contents to the target cell membrane. Here, we identify a small GTP-binding protein, ADP-ribosylation factor-like 8b (Arl8b), as a critical factor required for NK cell–mediated cytotoxicity. Our findings indicate that Arl8b drives the polarization of lytic granules and microtubule-organizing centers (MTOCs) toward the immune synapse between effector NK lymphocytes and target cells. Using a glutathione *S*-transferase pull-down approach, we identify kinesin family member 5B (KIF5B; the heavy chain of kinesin-1) as an interaction partner of Arl8b from NK cell lysates. Previous studies showed that interaction between kinesin-1 and Arl8b is mediated by SifA and kinesin-interacting protein (SKIP) and the tripartite complex drives the anterograde movement of lysosomes. Silencing of both KIF5B and SKIP in NK cells, similar to Arl8b, led to failure of MTOC-lytic granule polarization to the immune synapse, suggesting that Arl8b and kinesin-1 together control this critical step in NK cell cytotoxicity.

3.2096 Membrane-Anchored Aβ Accelerates Amyloid Formation and Exacerbates Amyloid-Associated Toxicity in Mice

Nagarathinam, A., Höfflinger, P., Bühler, A., Schäfer, C., McCovern, G., Jeffrey, M., Staufenbiel, M., Jucker, M. and Baumann, F.
J. Neurosci., ***33(49)***, *19284-19294 (2013)*

Pathological, genetic, and biochemical hallmarks of Alzheimer's disease (AD) are linked to amyloid-β (Aβ) peptide aggregation. Especially misfolded $A\beta_{42}$ peptide is sufficient to promote amyloid plaque formation. However, the cellular compartment facilitating the conversion of monomeric Aβ to aggregated toxic Aβ species remains unknown. *In vitro* models suggest lipid membranes to be the driving force of Aβ conversion. To this end, we generated two novel mouse models, expressing either membrane-anchored or nonanchored versions of the human $A\beta_{42}$ peptide. Strikingly, membrane-anchored $A\beta_{42}$ robustly accelerated Aβ deposition and exacerbated amyloid-associated toxicity upon crossing with Aβ precursor protein transgenic mice. These *in vivo* findings support the hypothesis that Aβ–membrane interactions play a pivotal role in early-onset AD as well as neuronal damage and provide evidence to study Aβ–membrane interactions as therapeutic targets.

3.2097 Genetic regulation of vesiculogenesis and immunomodulation in Mycobacterium tuberculosis

Rath, P., Huang, C., Wang, T., Wang, T., Li, H., Prados-Rosales, R., Elemento, O., Casadevall, A. and Nathan, C.F.
PNAS, ***110***, *E4790-E4797 (2013)*

Mycobacterium tuberculosis (Mtb) restrains immune responses well enough to escape eradication but elicits enough immunopathology to ensure its transmission. Here we provide evidence that this host–pathogen relationship is regulated in part by a cytosolic, membrane-associated protein with a unique structural fold, encoded by the Mtb gene *rv0431*. The protein acts by regulating the quantity of Mtb-derived membrane vesicles bearing Toll-like receptor 2 ligands, including the lipoproteins LpqH and SodC. We propose that *rv0431* be named “vesiculogenesis and immune response regulator.”

3.2098 **Impaired endolysosomal function disrupts Notch signalling in optic nerve astrocytes**
Valapala, M., Hose, S., Gongora, C., Dong, L., Wawrousek, E.F., Zigler Jr., J.S. and Sinha, D.
*Nature Communications, **4:**1629 (2013)*

Astrocytes migrate from the optic nerve into the inner retina, forming a template upon which retinal vessels develop. In the Nuc1 rat, mutation in the gene encoding βA3/A1-crystallin disrupts both Notch signalling in astrocytes and formation of the astrocyte template. Here we show that loss of βA3/A1-crystallin in astrocytes does not impede Notch ligand binding or extracellular cleavages. However, it affects vacuolar-type proton ATPase (V-ATPase) activity, thereby compromising acidification of the endolysosomal compartments, leading to reduced γ-secretase-mediated processing and release of the Notch intracellular domain (NICD). Lysosomal-mediated degradation of Notch is also impaired. These defects decrease the level of NICD in the nucleus, inhibiting the expression of Notch target genes. Overexpression of βA3/A1-crystallin in those same astrocytes restored V-ATPase activity and normal endolysosomal acidification, thereby increasing the levels of γ-secretase to facilitate optimal Notch signalling. We postulate that βA3/A1-crystallin is essential for normal endolysosomal acidification, and thereby, normal activation of Notch signalling in astrocytes.

3.2099 **Direct modulation of the outer mitochondrial membrane channel, voltage-dependent anion channel 1 (VDAC1) by cannabidiol: a novel mechanism for cannabinoid-induced cell death**
Rimmerman, N., Ben-Hail, D., Porat, Z., Juknat, A., Kozela, E., Daniels, M.P., Connelly, P.S., Leishman, E., Bradshaw, H.B., Shoshan-Barmatz, V. and Vogel, Z.
*Cell Death and Disease, **4**, e949 (2013)*

Cannabidiol (CBD) is a non-psychoactive plant cannabinoid that inhibits cell proliferation and induces cell death of cancer cells and activated immune cells. It is not an agonist of the classical CB1/CB2 cannabinoid receptors and the mechanism by which it functions is unknown. Here, we studied the effects of CBD on various mitochondrial functions in BV-2 microglial cells. Our findings indicate that CBD treatment leads to a biphasic increase in intracellular calcium levels and to changes in mitochondrial function and morphology leading to cell death. Density gradient fractionation analysis by mass spectrometry and western blotting showed colocalization of CBD with protein markers of mitochondria. Single-channel recordings of the outer-mitochondrial membrane protein, the voltage-dependent anion channel 1 (VDAC1) functioning in cell energy, metabolic homeostasis and apoptosis revealed that CBD markedly decreases channel conductance. Finally, using microscale thermophoresis, we showed a direct interaction between purified fluorescently labeled VDAC1 and CBD. Thus, VDAC1 seems to serve as a novel mitochondrial target for CBD. The inhibition of VDAC1 by CBD may be responsible for the immunosuppressive and anticancer effects of CBD.

3.2100 **Interaction of ganglioside GD3 with an EGF receptor sustains the self-renewal ability of mouse neural stem cells in vitro**
Wang, J. and Yu, R.K.
*PNAS, **110(47)**, 19137-19142 (2013)*

Mounting evidence supports the notion that gangliosides serve regulatory roles in neurogenesis; little is known, however, about how these glycosphingolipids function in neural stem cell (NSC) fate determination. We previously demonstrated that ganglioside GD3 is a major species in embryonic mouse brain: more than 80% of the NSCs obtained by the neurosphere method express GD3. To investigate the functional role of GD3 in neurogenesis, we compared the properties of NSCs from GD3-synthase knockout (GD3S-KO) mice with those from their wild-type littermates. NSCs from GD3S-KO mice showed decreased self-renewal ability compared with those from the wild-type animals, and that decreased ability was accompanied by reduced expression of EGF receptor (EGFR) and an increased degradation rate of EGFR and EGF-induced ERK signaling. We also showed that EGFR switched from the low-density lipid raft fractions in wild-type NSCs to the high-density layers in the GD3S-KO NSCs. Immunochemical staining revealed colocalization of EGFR and GD3, and EGFR could be immunoprecipitated from the NSC lysate with an anti-GD3 antibody from the wild-type, but not from the GD3S-KO, mice. Tracking the localization of endocytosed EGFR with endocytosis pathway markers indicated that more EGFR in GD3S-KO NSCs translocated through the endosomal−lysosomal degradative pathway, rather than through the recycling pathway. Those findings support the idea that GD3 interacts with EGFR in the NSCs and that the interaction is responsible for sustaining the expression of EGFR and its downstream signaling to maintain the self-renewal capability of NSCs.

3.2101 RNA-sequencing from single nuclei

Grindberg, R.V., Yee-Greenbaum, J.L., McConnell, M.J., Novotny, M., O'Shaugnessy, A.L., Lambert, G.M., Arauzo-Bravo, M.J., Lee, J., Fishman, M., Robbins, G.E., Lin, X., Venepally, P., Badger, J.H., Galbraith, D.W., Gage, F.H. and Lasken, R.S.
*PNAS, **110(49)**, 19802-19807 (2013)*

It has recently been established that synthesis of double-stranded cDNA can be done from a single cell for use in DNA sequencing. Global gene expression can be quantified from the number of reads mapping to each gene, and mutations and mRNA splicing variants determined from the sequence reads. Here we demonstrate that this method of transcriptomic analysis can be done using the extremely low levels of mRNA in a single nucleus, isolated from a mouse neural progenitor cell line and from dissected hippocampal tissue. This method is characterized by excellent coverage and technical reproducibility. On average, more than 16,000 of the 24,057 mouse protein-coding genes were detected from single nuclei, and the amount of gene-expression variation was similar when measured between single nuclei and single cells. Several major advantages of the method exist: first, nuclei, compared with whole cells, have the advantage of being easily isolated from complex tissues and organs, such as those in the CNS. Second, the method can be widely applied to eukaryotic species, including those of different kingdoms. The method also provides insight into regulatory mechanisms specific to the nucleus. Finally, the method enables dissection of regulatory events at the single-cell level; pooling of 10 nuclei or 10 cells obscures some of the variability measured in transcript levels, implying that single nuclei and cells will be extremely useful in revealing the physiological state and interconnectedness of gene regulation in a manner that avoids the masking inherent to conventional transcriptomics using bulk cells or tissues.

3.2102 Bioinspired Exosome-Mimetic Nanovesicles for Targeted Delivery of Chemotherapeutics to Malignant Tumors

Jang, S.C., Kim, O.Y., Yoon, C.M., Choi, D-S., Roh, T-Y., park, J., Nilsson, J., Lötvall, J., Kim, Y-K. and Gho, Y.S.
*ACSNano, **7(9)**, 7698-7710 (2013)*

Exosomes, the endogenous nanocarriers that can deliver biological information between cells, were recently introduced as new kind of drug delivery system. However, mammalian cells release relatively low quantities of exosomes, and purification of exosomes is difficult. Here, we developed bioinspired exosome-mimetic nanovesicles that deliver chemotherapeutics to the tumor tissue after systemic administration. The chemotherapeutics-loaded nanovesicles were produced by the breakdown of monocytes or macrophages using a serial extrusion through filters with diminishing pore sizes (10, 5, and 1 μm). These cell-derived nanovesicles have similar characteristics with the exosomes but have 100-fold higher production yield. Furthermore, the nanovesicles have natural targeting ability of cells by maintaining the topology of plasma membrane proteins. *In vitro*, chemotherapeutic drug-loaded nanovesicles induced TNF-α-stimulated endothelial cell death in a dose-dependent manner. *In vivo*, experiments in mice showed that the chemotherapeutic drug-loaded nanovesicles traffic to tumor tissue and reduce tumor growth without the adverse effects observed with equipotent free drug. Furthermore, compared with doxorubicin-loaded exosomes, doxorubicin-loaded nanovesicles showed similar *in vivo* antitumor activity. However, doxorubicin-loaded liposomes that did not carry targeting proteins were inefficient in reducing tumor growth. Importantly, removal of the plasma membrane proteins by trypsinization eliminated the therapeutic effects of the nanovesicles both *in vitro* and *in vivo*. Taken together, these studies suggest that the bioengineered nanovesicles can serve as novel exosome-mimetics to effectively deliver chemotherapeutics to treat malignant tumors.

3.2103 Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma

Kalra, H., Adda, C.G., Liem, M., Ang, C-S., Mechler, A., Simpson, R.J., Hulett, M.D. and Mathivanan, S.
*Proteomics, **13(22)**, 3354-3364 (2013)*

Exosomes are nanovesicles released by a variety of cells and are detected in body fluids including blood. Recent studies have highlighted the critical application of exosomes as personalized targeted drug delivery vehicles and as reservoirs of disease biomarkers. While these research applications have created significant interest and can be translated into practice, the stability of exosomes needs to be assessed and exosome isolation protocols from blood plasma need to be optimized. To optimize methods to isolate exosomes from blood plasma, we performed a comparative evaluation of three exosome isolation techniques (differential centrifugation coupled with ultracentrifugation, epithelial cell adhesion molecule

immunoaffinity pull-down, and OptiPrep™ density gradient separation) using normal human plasma. Based on MS, Western blotting and microscopy results, we found that the OptiPrep™ density gradient method was superior in isolating pure exosomal populations, devoid of highly abundant plasma proteins. In addition, we assessed the stability of exosomes in plasma over 90 days under various storage conditions. Western blotting analysis using the exosomal marker, TSG101, revealed that exosomes are stable for 90 days. Interestingly, in the context of cellular uptake, the isolated exosomes were able to fuse with target cells revealing that they were indeed biologically active.

3.2104 EGFR inhibitor BIBU induces apoptosis and defective autophagy in glioma cells
Ghildiyal, R., Dixit, D. and Sen, E.
*Mol. Carcinogenesis, **52(12)**, 970-982 (2013)*

The importance of aberrant EGFR signaling in glioblastoma progression and the promise of EGFR-specific therapies, prompted us to determine the efficacy of novel EGFR inhibitor BIBU-1361 [(3-chloro-4-fluoro-phenyl)-[6-(4-diethylaminomethyl-piperidin-1-yl)-pyrimido [5,4-*d*]pyrimidin-4-yl]-amine] in affecting glioma survival. BIBU induced apoptosis in a caspase-dependent manner and induced cell cycle arrest in glioma cells. Apoptosis was accompanied by decreased EGFR levels and its increased distribution towards caveolin rich lipid raft microdomains. BIBU inhibited pro-survival pathways Akt/mTOR and gp130/JAK/STAT3; and decreased levels of pro-inflammatory cytokine IL-6. BIBU caused increased LC3-I to LC3-II conversion and triggered the internalization of EGFR within vacuoles along with its increased co-localization with LC3-II. BIBU caused accumulation of p62 and increased levels of cleaved forms of Beclin-1 in all the cell lines tested. Importantly, BIBU failed to initiate execution of autophagy as pharmacological inhibition of autophagy with 3-Methyladenine or Bafilomycin failed to rescue BIBU mediated death. The ability of BIBU to abrogate Akt and STAT3 activation, induce apoptosis and prevent execution of autophagy warrants its investigation as a potent anti-glioma target

3.2105 Enterohemorrhagic Escherichia coli Hemolysin Employs Outer Membrane Vesicles to Target Mitochondria and Cause Endothelial and Epithelial Apoptosis
Bielaszewska, M., Rüter, C., Kunsmann, L., Greune, L., Bauwens, A., Zhang, W., Kuczius, T., Kim, K.S., Mellmann, A., Schmidt, M.A.. and Karch, H.
*PloS Pathogens, **9(12)**, e1003797 (2013)*

Enterohemorrhagic *Escherichia coli* (EHEC) strains cause diarrhea and hemolytic uremic syndrome resulting from toxin-mediated microvascular endothelial injury. EHEC hemolysin (EHEC-Hly), a member of the RTX (repeats-in-toxin) family, is an EHEC virulence factor of increasingly recognized importance. The toxin exists as free EHEC-Hly and as EHEC-Hly associated with outer membrane vesicles (OMVs) released by EHEC during growth. Whereas the free toxin is lytic towards human endothelium, the biological effects of the OMV-associated EHEC-Hly on microvascular endothelial and intestinal epithelial cells, which are the major targets during EHEC infection, are unknown. Using microscopic, biochemical, flow cytometry and functional analyses of human brain microvascular endothelial cells (HBMEC) and Caco-2 cells we demonstrate that OMV-associated EHEC-Hly does not lyse the target cells but triggers their apoptosis. The OMV-associated toxin is internalized by HBMEC and Caco-2 cells via dynamin-dependent endocytosis of OMVs and trafficked with OMVs into endo-lysosomal compartments. Upon endosome acidification and subsequent pH drop, EHEC-Hly is separated from OMVs, escapes from the lysosomes, most probably via its pore-forming activity, and targets mitochondria. This results in decrease of the mitochondrial transmembrane potential and translocation of cytochrome c to the cytosol, indicating EHEC-Hly-mediated permeabilization of the mitochondrial membranes. Subsequent activation of caspase-9 and caspase-3 leads to apoptotic cell death as evidenced by DNA fragmentation and chromatin condensation in the intoxicated cells. The ability of OMV-associated EHEC-Hly to trigger the mitochondrial apoptotic pathway in human microvascular endothelial and intestinal epithelial cells indicates a novel mechanism of EHEC-Hly involvement in the pathogenesis of EHEC diseases. The OMV-mediated intracellular delivery represents a newly recognized mechanism for a bacterial toxin to enter host cells in order to target mitochondria.

3.2106 A Hereditary Spastic Paraplegia Mouse Model Supports a Role of ZFYVE26/SPASTIZIN for the Endolysosomal System
Khundadze, M., Kollmann, K., Koch, N., Biskup, C., Nietzsche, S., Zimmer, G., Hennings, J.C., Huebner, A.K., Symmank, J., Jahic, A., Ilina, E.I., Karle, K., Schöls, L., Kessels, M., Braulke, T., Qualmann, B., Kurth, I., Beetz, C. and Hübner, C.A.
*PloS Genetics, **9(12)**, e1003988 (2013)*

Hereditary spastic paraplegias (HSPs) are characterized by progressive weakness and spasticity of the legs because of the degeneration of cortical motoneuron axons. SPG15 is a recessively inherited HSP variant caused by mutations in the *ZFYVE26* gene and is additionally characterized by cerebellar ataxia, mental decline, and progressive thinning of the corpus callosum. *ZFYVE26* encodes the FYVE domain-containing protein ZFYVE26/SPASTIZIN, which has been suggested to be associated with the newly discovered adaptor protein 5 (AP5) complex. We show that Zfyve26 is broadly expressed in neurons, associates with intracellular vesicles immunopositive for the early endosomal marker EEA1, and co-fractionates with a component of the AP5 complex. As the function of ZFYVE26 in neurons was largely unknown, we disrupted *Zfyve26* in mice. *Zfyve26* knockout mice do not show developmental defects but develop late-onset spastic paraplegia with cerebellar ataxia confirming that SPG15 is caused by ZFYVE26 deficiency. The morphological analysis reveals axon degeneration and progressive loss of both cortical motoneurons and Purkinje cells in the cerebellum. Importantly, neuron loss is preceded by accumulation of large intraneuronal deposits of membrane-surrounded material, which co-stains with the lysosomal marker Lamp1. A density gradient analysis of brain lysates shows an increase of Lamp1-positive membrane compartments with higher densities in *Zfyve26* knockout mice. Increased levels of lysosomal enzymes in brains of aged knockout mice further support an alteration of the lysosomal compartment upon disruption of *Zfyve26*. We propose that SPG15 is caused by an endolysosomal membrane trafficking defect, which results in endolysosomal dysfunction. This appears to be particularly relevant in neurons with highly specialized neurites such as cortical motoneurons and Purkinje cells.

3.2107 Lysosomal NEU1 deficiency affects amyloid precursor protein levels and amyloid-β secretion via deregulated lysosomal exocytosis

Annunziata, I., Patterson, A., Helton, D., Hu, H., Moshiach, S., Gomero, e., Nixon, R. and d'Azzo, A.
*Nature Communications, **4:**2734 (2013)*

Alzheimer's disease (AD) belongs to a category of adult neurodegenerative conditions, which are associated with intracellular and extracellular accumulation of neurotoxic protein aggregates. Understanding how these aggregates are formed, secreted and propagated by neurons has been the subject of intensive research, but so far no preventive or curative therapy for AD is available, and clinical trials have been largely unsuccessful. Here we show that deficiency of the lysosomal sialidase NEU1 leads to the spontaneous occurrence of an AD-like amyloidogenic process in mice. This involves two consecutive events linked to NEU1 loss-of-function—accumulation and amyloidogenic processing of an oversialylated amyloid precursor protein in lysosomes, and extracellular release of Aβ peptides by excessive lysosomal exocytosis. Furthermore, cerebral injection of NEU1 in an established AD mouse model substantially reduces β-amyloid plaques. Our findings identify an additional pathway for the secretion of Aβ and define NEU1 as a potential therapeutic molecule for AD.

3.2108 sCD44 overexpression increases intraocular pressure and aqueous outflow resistance

Giovingo, M., Nolan, M., McCarty, r., Pang, I-H., Clark, A.F., Beverley, R.M., Schwartz, S., Stamer, W.D., Walker, L., Grybauskas, A., Skuran, K., Kuprys, P.V., Yue, B.Y.J.T. and Knepper, P.A.
*Mol. Vis., **19**, 2151-2164 (2013)*

PURPOSE:
CD44 plays major roles in multiple physiologic processes. The ectodomain concentration of the CD44 receptor, soluble CD44 (sCD44), is significantly increased in the aqueous humor of primary open-angle glaucoma (POAG). The purpose of this study was to determine if adenoviral constructs of CD44 and isolated 32-kDa sCD44 change intraocular pressure (IOP) in vivo and aqueous outflow resistance in vitro.
METHODS:
Adenoviral constructs of human standard CD44 (Ad-CD44S), soluble CD44 (Ad-sCD44), and empty viral cDNA were injected into the vitreous of BALB/cJ mice, followed by serial IOP measurements. Overexpression of CD44S and sCD44 was verified in vitro by enzyme-linked immunosorbent assay (ELISA) and western blot analysis. Anterior segments of porcine eyes were perfused with the isolated sCD44. sCD44-treated human trabecular meshwork (TM) cells and microdissected porcine TM were examined by confocal microscopy and Optiprep density gradient with western blot analysis to determine changes in lipid raft components.
RESULTS:
Intravitreous injection of adenoviral constructs with either Ad-CD44S or Ad-sCD44 vectors caused prolonged ocular hypertension in mice. Eight days after vector injection, Ad-CD44S significantly elevated IOP to 28.3±1.2 mmHg (mean±SEM, n=8; $p<0.001$); Ad-sCD44 increased IOP to 18.5±2.6 mmHg (n=8;

p<0.01), whereas the IOP of uninjected eyes was 12.7±0.2 mmHg (n=16). The IOP elevation lasted more than 50 days. Topical administration of a γ-secretase inhibitor normalized Ad-sCD44-induced elevated IOP. sCD44 levels were significantly elevated in the aqueous humor of Ad-CD44S and Ad-sCD44 eyes versus contralateral uninjected eyes (p<0.01). Anterior segment perfusion of isolated 32-kDa sCD44 significantly decreased aqueous outflow rates. Co-administration of isolated sCD44 and CD44 neutralizing antibody or of γ-secretase inhibitor significantly enhanced flow rates. sCD44-treated human TM cells displayed cross-linked actin network formation. Optiprep density gradient and western blot analysis of human TM cells treated with sCD44 showed decreased annexin 2 expression and increased phosphorylated annexin 2 and caveolin 1 expression.

CONCLUSIONS:

Our data suggest that sCD44 increases outflow resistance in vivo and in vitro. Viral overexpression of both CD44S and sCD44 is sufficient to cause ocular hypertension. Infusion of sCD44 in porcine anterior segment eyes significantly decreased flow rates. Notably, sCD44 enhanced cross-linked actin network formation. The elevated sCD44 levels seen in POAG aqueous humor may play an important causative role in POAG pathogenesis.

3.2109 Viral Attachment Induces Rapid Recruitment of an Innate Immune Sensor (TRIM5α) to the Plasma Membrane

Ohmine, S., Singh, R.D., Marks, D.L., Meyer, M.A., Pagano, R. and Ukeda, Y.
J. Innate Immun., ***5***, *414-424 (2013)*

TRIM5α (tripartite motif 5α) acts as a pattern recognition receptor specific for the retrovirus capsid lattice and blocks infection by HIV-1 immediately after entry. However, the precise mechanisms underlying this rapid recognition of viral components remain elusive. Here, we analyzed the influence of viral exposure on TRIM5α. Total internal reflection fluorescence microscopy and lipid flotation assays revealed rapid recruitment of a TRIM5α subpopulation to the plasma membrane (PM) upon exposure to vesicular stomatitis virus-G-pseudotyped HIV-1 viral-like particles (VLPs), but not to envelope (Env)-less HIV-1 VLPs. TRIM5α signals were frequently colocalized with those of HIV-1 capsid at the PM. Exposure to HIV-1 Env-pseudotyped HIV-1 vectors also triggered translocation of endogenous TRIM5α to lipid microdomains within human T cells. Similarly, clustering of lipid microdomains by a glycosphingolipid stereoisomer resulted in rapid TRIM5α recruitment to the PM. Of note, recruitment of endogenous rhesus TRIM5α to the PM prior to HIV-1 infection significantly increased the potency of viral restriction. Our data therefore suggest the importance of TRIM5α recruitment to the PM for TRIM5α-mediated innate immune sensing and restriction of retroviral infection.

3.2110 The Role of Ect2 Nuclear RhoGEF Activity in Ovarian Cancer Cell Transformation

Huff, L.P., DeCristo, M.J., Trembath, D., Kuan, P.F., Yim, M., Liu, J., Cook, D.R., Miller, C.R., Der, C.J. and Cox, A.D.
Genes & Cancer, ***4(11-12)****, 460-475 (2013)*

Ect2, a Rho guanine nucleotide exchange factor (RhoGEF), is atypical among RhoGEFs in its predominantly nuclear localization in interphase cells. One current model suggests that Ect2 mislocalization drives cellular transformation by promoting aberrant activation of cytoplasmic Rho family GTPase substrates. However, in ovarian cancers, where Ect2 is both amplified and overexpressed at the mRNA level, we observed that the protein is highly expressed and predominantly nuclear and that nuclear but not cytoplasmic Ect2 increases with advanced disease. Knockdown of Ect2 in ovarian cancer cell lines impaired their anchorage-independent growth without affecting their growth on plastic. Restoration of Ect2 expression rescued the anchorage-independent growth defect, but not if either the DH catalytic domain or the nuclear localization sequences of Ect2 were mutated. These results suggested a novel mechanism whereby Ect2 could drive transformation in ovarian cancer cells by acting as a RhoGEF specifically within the nucleus. Interestingly, Ect2 had an intrinsically distinct GTPase specificity profile in the nucleus versus the cytoplasm. Nuclear Ect2 bound preferentially to Rac1, while cytoplasmic Ect2 bound to RhoA but not Rac. Consistent with nuclear activation of endogenous Rac, Ect2 overexpression was sufficient to recruit Rac effectors to the nucleus, a process that required a functional Ect2 catalytic domain. Furthermore, expression of active nuclearly targeted Rac1 rescued the defect in transformed growth caused by Ect2 knockdown. Our work suggests a novel mechanism of Ect2-driven transformation, identifies subcellular localization as a regulator of GEF specificity, and implicates activation of nuclear Rac1 in cellular transformation.

3.2111 Thrombomodulin functions as a plasminogen receptor to modulate angiogenesis
Chen, P-K., Chang, B-I., Kuo, C-H., Chen, P-S., Cho, C-F., Chang, C-F., Shi, G-Y. and Wu, H-L.
*FASEB J., **27(11)**, 4520-4531 82013)*

Urokinase-type plasminogen activator (uPA) activates plasminogen (Plg) through a major pericellular proteolytic system involved in cell migration and angiogenesis; however, the Plg receptor that participates in uPA-mediated Plg activation has not yet been identified. In this study, we demonstrated that thrombomodulin (TM), a type I transmembrane glycoprotein, is a novel Plg receptor that plays a role in pericellular proteolysis and cell migration. Plg activation at the cell surface and the extent of its cell migration- and invasion-promoting effect are cellular TM expression dependent. Direct binding of Plg and the recombinant TM extracellular domain, with a K_D of 0.1−0.3 μM, was determined through surface plasmon resonance analysis. Colocalization of TM, Plg, and the uPA receptor within plasma membrane lipid rafts, at the leading edge of migrating endothelial cells, was demonstrated and was also shown to overlap with areas of major pericellular proteolysis. Moreover, the roles of TM and Plg in neoangiogenesis were demonstrated *in vivo* through the skin wound-healing model. In conclusion, we propose that TM is a novel Plg receptor that regulates uPA/uPA receptor-mediated Plg activation and pericellular proteolysis within lipid rafts at the leading edge of migrating cells during angiogenesis.

3.2112 Differential Association of the Na+/H+ Exchanger Regulatory Factor (NHERF) Family of Adaptor Proteins with the Raft- and the Non-Raft Brush Border Membrane Fractions of NHE3
Sultan, A., Luo, M., Yu, Q., Riederer, B., Xia, W., Chen, M., Lissner, S., Gessner, J.E., Donowitz, M., Yun, C.C., deJonge, H., Lamprecht, G. and Seidler, U.
*Cell. Physiol. Biochem., **32(5)**, 1386-1402 (2013)*

Background/Aims: Trafficking, brush border membrane (BBM) retention, and signal-specific regulation of the Na^+/H^+ exchanger NHE3 is regulated by the Na^+/H^+ Exchanger Regulatory Factor (NHERF) family of PDZ-adaptor proteins, which enable the formation of multiprotein complexes. It is unclear, however, what determines signal specificity of these NHERFs. Thus, we studied the association of NHE3, NHERF1 (EBP50), NHERF2 (E3KARP), and NHERF3 (PDZK1) with lipid rafts in murine small intestinal BBM. ***Methods:*** Detergent resistant membranes ("lipid rafts") were isolated by floatation of Triton X-incubated small intestinal BBM from a variety of knockout mouse strains in an Optiprep step gradient. Acid-activated NHE3 activity was measured fluorometrically in BCECF-loaded microdissected villi, or by assessment of CO_2/HCO_3^- mediated increase in fluid absorption in perfused jejunal loops of anethetized mice. ***Results:*** NHE3 was found to partially associate with lipid rafts in the native BBM, and NHE3 raft association had an impact on NHE3 transport activity and regulation *in vivo*. NHERF1, 2 and 3 were differentially distributed to rafts and non-rafts, with NHERF2 being most raft-associated and NHERF3 entirely non-raft associated. NHERF2 expression enhanced the localization of NHE3 to membrane rafts. The use of acid sphingomyelinase-deficient mice, which have altered membrane lipid as well as lipid raft composition, allowed us to test the validity of the lipid raft concept *in vivo*. ***Conclusions:*** The differential association of the NHERFs with the raft-associated and the non-raft fraction of NHE3 in the brush border membrane is one component of the differential and signal-specific NHE3 regulation by the different NHERFs.

3.2113 Autophagy proteins stabilize pathogen-containing phagosomes for prolonged MHC II antigen processing
Romao, S., Gasser, N., Becker, A.C., Guhl, B., Bajagic, M., Vanoaica, D., Ziegler, U., Roesler, J., Dengjel, J., Reichenbach, J. and Münz, C.
J. Cell Biol., 203(5), 757-766 (2013)

Antigen preservation for presentation is a hallmark of potent antigen-presenting cells. In this paper, we report that in human macrophages and dendritic cells, a subset of phagosomes gets coated with Atg8/LC3, a component of the molecular machinery of macroautophagy, and maintains phagocytosed antigens for prolonged presentation on major histocompatibility complex class II molecules. These Atg8/LC3-positive phagosomes are formed around the antigen with TLR2 agonists and require reactive oxygen species production by NOX2 for their generation. A deficiency in the NOX2-dependent formation of these antigen storage phagosomes could contribute to compromise antifungal immune control in chronic granulomatous disease patients

3.2114 Interaction of membrane/lipid rafts with the cytoskeleton: Impact on signaling and function ☆: Membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling
Head, B.P., Patel, H.H. and Insel, P.A.

*Biochim. Biophys. Acta, **1838**, 532-545 (2014)*

The plasma membrane in eukaryotic cells contains microdomains that are enriched in certain glycosphingolipids, gangliosides, and sterols (such as cholesterol) to form membrane/lipid rafts (MLR). These regions exist as caveolae, morphologically observable flask-like invaginations, or as a less easily detectable planar form. MLR are scaffolds for many molecular entities, including signaling receptors and ion channels that communicate extracellular stimuli to the intracellular milieu. Much evidence indicates that this organization and/or the clustering of MLR into more active signaling platforms depends upon interactions with and dynamic rearrangement of the cytoskeleton. Several cytoskeletal components and binding partners, as well as enzymes that regulate the cytoskeleton, localize to MLR and help regulate lateral diffusion of membrane proteins and lipids in response to extracellular events (e.g., receptor activation, shear stress, electrical conductance, and nutrient demand). MLR regulate cellular polarity, adherence to the extracellular matrix, signaling events (including ones that affect growth and migration), and are sites of cellular entry of certain pathogens, toxins and nanoparticles. The dynamic interaction between MLR and the underlying cytoskeleton thus regulates many facets of the function of eukaryotic cells and their adaptation to changing environments. Here, we review general features of MLR and caveolae and their role in several aspects of cellular function, including polarity of endothelial and epithelial cells, cell migration, mechanotransduction, lymphocyte activation, neuronal growth and signaling, and a variety of disease settings. This article is part of a Special Issue entitled: Reciprocal influences between cell cytoskeleton and membrane channels, receptors and transporters.

3.2115 Differentially localized acyl-CoA synthetase 4 isoenzymes mediate the metabolic channeling of fatty acids towards phosphatidylinositol
Küch, E-M., Vellaramkalayil, R., Zhang, I., Lehnen, D., Brügger, B., Stremmel, W., Ehehalt, R., Poppelreuther, M. and Füllekrug, J.
*Biochem. Biophys. Acta, **1841**, 227-239 (2013)*

The acyl-CoA synthetase 4 (ACSL4) has been implicated in carcinogenesis and neuronal development. Acyl-CoA synthetases are essential enzymes of lipid metabolism, and ACSL4 is distinguished by its preference for arachidonic acid. Two human ACSL4 isoforms arising from differential splicing were analyzed by ectopic expression in COS cells. We found that the ACSL4_v1 variant localized to the inner side of the plasma membrane including microvilli, and was also present in the cytosol. ACSL4_v2 contains an additional N-terminal hydrophobic region; this isoform was located at the endoplasmic reticulum and on lipid droplets. A third isoform was designed de novo by appending a mitochondrial targeting signal. All three ACSL4 variants showed the same specific enzyme activity. Overexpression of the isoenzymes increased cellular uptake of arachidonate to the same degree, indicating that the metabolic trapping of fatty acids is independent of the subcellular localization. Remarkably, phospholipid metabolism was changed by ACSL4 expression. Labeling with arachidonate showed that the amount of newly synthesized phosphatidylinositol was increased by all three ACSL4 isoenzymes but not by ACSL1. This was dependent on the expression level and the localization of the ACSL4 isoform. We conclude that in our model system exogenous fatty acids are channeled preferentially towards phosphatidylinositol by ACSL4 overexpression. The differential localization of the endogenous isoenzymes may provide compartment specific precursors of this anionic phospholipid important for many signaling processes.

3.2116 Prolonged Insulin Stimulation Down-regulates GLUT4 through Oxidative Stress-mediated Retromer Inhibition by a Protein Kinase CK2-dependent Mechanism in 3T3-L1 Adipocytes
Ma, J., Nakagawa, Y., Kojima, I. and Shibata, H.
*J. Biol. Chem., **289(1)**, 133-142 (2014)*

Although insulin acutely stimulates glucose uptake by promotion of GLUT4 translocation from intracellular compartments to the plasma membrane in adipocytes and muscles, long term insulin stimulation causes GLUT4 depletion that is particularly prominent in the insulin-responsive GLUT4 storage compartment. This effect is caused mainly by accelerated lysosomal degradation of GLUT4, although the mechanism is not fully defined. Here we show that insulin acutely induced dissociation of retromer components from the low density microsomal membranes of 3T3-L1 adipocytes that was accompanied by disruption of the interaction of Vps35 with sortilin. This insulin effect was dependent on the activity of protein kinase CK2 but not phosphatidylinositol 3-kinase or extracellular signal-regulated kinase 1/2. Knockdown of Vps26 decreased GLUT4 to a level comparable with that with insulin stimulation for 4 h. Vps35 with a mutation in the CK2 phosphorylation motif (Vps35-S7A) was resistant to insulin-induced dissociation from the low density microsomal membrane, and its overexpression

attenuated GLUT4 down-regulation with insulin. Furthermore, insulin-generated hydrogen peroxide was an upstream mediator of the insulin action on retromer and GLUT4. These results suggested that insulin-generated oxidative stress switches the GLUT4 sorting direction to lysosomes through inhibition of the retromer function in a CK2-dependent manner.

3.2117 Pseudomonas aeruginosa Cif Protein Enhances the Ubiquitination and Proteasomal Degradation of the Transporter Associated with Antigen Processing (TAP) and Reduces Major Histocompatibility Complex (MHC) Class I Antigen Presentation

Bomberger, J.M., Ely, K.H., Bangia, N., Ye, S., Green, K.A., Green, W.R., Enelow, R.I. and Stanton, B.A.
J. Biol. Chem., ***289(1)***, *152-162 (2014)*

Cif (PA2934), a bacterial virulence factor secreted in outer membrane vesicles by *Pseudomonas aeruginosa*, increases the ubiquitination and lysosomal degradation of some, but not all, plasma membrane ATP-binding cassette transporters (ABC), including the cystic fibrosis transmembrane conductance regulator and P-glycoprotein. The goal of this study was to determine whether Cif enhances the ubiquitination and degradation of the transporter associated with antigen processing (TAP1 and TAP2), members of the ABC transporter family that play an essential role in antigen presentation and intracellular pathogen clearance. Cif selectively increased the amount of ubiquitinated TAP1 and increased its degradation in the proteasome of human airway epithelial cells. This effect of Cif was mediated by reducing USP10 deubiquitinating activity, resulting in increased polyubiquitination and proteasomal degradation of TAP1. The reduction in TAP1 abundance decreased peptide antigen translocation into the endoplasmic reticulum, an effect that resulted in reduced antigen available to MHC class I molecules for presentation at the plasma membrane of airway epithelial cells and recognition by $CD8^+$ T cells. Cif is the first bacterial factor identified that inhibits TAP function and MHC class I antigen presentation.

3.2118 Angiopoietin-2 Secretion by Endothelial Cell Exosomes: REGULATION BY THE PHOSPHATIDYLINOSITOL 3-KINASE (PI3K)/Akt/ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) AND SYNDECAN-4/SYNTENIN PATHWAYS

Ju, R., Zhuang, Z.W., Zhang, J., Lanahan, A.A., Kyriakides, T., Sessa, W.C. and Simons, M.
J. Biol. Chem., ***289(1)***, *510-519 (2014)*

Angiopoietin-2 (Ang2) is an extracellular protein and one of the principal ligands of Tie2 receptor that is involved in the regulation of vascular integrity, quiescence, and inflammation. The mode of secretion of Ang2 has never been established, however. Here, we provide evidence that Ang2 is secreted from endothelial cells via exosomes and that this process is inhibited by the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway, whereas it is positively regulated by the syndecan-4/syntenin pathway. Vascular defects in Akt1 null mice arise, in part, because of excessive Ang2 secretion and can be rescued by the syndecan-4 knock-out that reduces extracellular Ang2 levels. This novel mechanism connects three critical signaling pathways: angiopoietin/Tie2, PI3K/Akt/eNOS, and syndecan/syntenin, which play important roles in vascular growth and stabilization.

3.2119 Phospholipase D2 Mediates Survival Signaling through Direct Regulation of Akt in Glioblastoma Cells

Bruntz, R.C., Taylor, H.E., Lindsley, C.W. and Brown, H.A.
J. Biol. Chem., ***289(2)***, *600-616 (2014)*

The lack of innovative drug targets for glioblastoma multiforme (GBM) limits patient survival to approximately 1 year following diagnosis. The pro-survival kinase Akt provides an ideal target for the treatment of GBM as Akt signaling is frequently activated in this cancer type. However, the central role of Akt in physiological processes limits its potential as a therapeutic target. In this report, we show that the lipid-metabolizing enzyme phospholipase D (PLD) is a novel regulator of Akt in GBM. Studies using a combination of small molecule PLD inhibitors and siRNA knockdowns establish phosphatidic acid, the product of the PLD reaction, as an essential component for the membrane recruitment and activation of Akt. Inhibition of PLD enzymatic activity and subsequent Akt activation decreases GBM cell viability by specifically inhibiting autophagic flux. We propose a mechanism whereby phosphorylation of beclin1 by Akt prevents binding of Rubicon (RUN domain cysteine-rich domain containing beclin1-interacting protein), an interaction known to inhibit autophagic flux. These findings provide a novel framework through which Akt inhibition can be achieved without directly targeting the kinase.

3.2120 **Disruption of the Coxsackievirus and Adenovirus Receptor-Homodimeric Interaction Triggers Lipid Microdomain- and Dynamin-dependent Endocytosis and Lysosomal Targeting**
Salinas, S., Zussy, C., Loustalot, F., Henaff, D., Menendez, G., Morton, P.E., Parsons, M., Schiavo, G. and Kremer, E.J.
J. Biol. Chem., ***289(2)***, *680-695 (2014)*

The coxsackievirus and adenovirus receptor (CAR) serves as a docking factor for some adenovirus (AdV) types and group B coxsackieviruses. Its role in AdV internalization is unclear as studies suggest that its intracellular domain is dispensable for some AdV infection. We previously showed that in motor neurons, AdV induced CAR internalization and co-transport in axons, suggesting that CAR was linked to endocytic and long-range transport machineries. Here, we characterized the mechanisms of CAR endocytosis in neurons and neuronal cells. We found that CAR internalization was lipid microdomain-, actin-, and dynamin-dependent, and subsequently followed by CAR degradation in lysosomes. Moreover, ligands that disrupted the homodimeric CAR interactions in its D1 domains triggered an internalization cascade involving sequences in its intracellular tail.

3.2121 **Prion Infection Impairs Cholesterol Metabolism in Neuronal Cells**
Cui, H., Guo, B., Scicluna, B., Coleman, B.M., Lawson, V.A., Ellett, L., Meikle, P.J., Bukrinsky, M., Mukhamedova, N., Sviridov, D. and Hill, A.F.
J. Biol. Chem., ***289(2)***, *789-802 (2014)*

Conversion of prion protein (PrP^{C}) into a pathological isoform (PrP^{Sc}) during prion infection occurs in lipid rafts and is dependent on cholesterol. Here, we show that prion infection increases the abundance of cholesterol transporter, ATP-binding cassette transporter type A1 (ATP-binding cassette transporter type A1), but reduces cholesterol efflux from neuronal cells leading to the accumulation of cellular cholesterol. Increased abundance of ABCA1 in prion disease was confirmed in prion-infected mice. Mechanistically, conversion of PrP^{C} to the pathological isoform led to PrP^{Sc} accumulation in rafts, displacement of ABCA1 from rafts and the cell surface, and enhanced internalization of ABCA1. These effects were abolished with reversal of prion infection or by loading cells with cholesterol. Stimulation of ABCA1 expression with liver X receptor agonist or overexpression of heterologous ABCA1 reduced the conversion of prion protein into the pathological form upon infection. These findings demonstrate a reciprocal connection between prion infection and cellular cholesterol metabolism, which plays an important role in the pathogenesis of prion infection in neuronal cells.

3.2122 **Inflammation, caveolae and CD38-mediated calcium regulation in human airway smooth muscle**
Sathish, V., Thompson, M.A., Sinha, S., Sieck, G.C., Prakash, Y.S. and Pabelick, C.M:
Biochem. Biophys. Acta, ***1843***, *346-351 (2014)*

The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF α) increases expression of CD38 (a membrane-associated bifunctional enzyme regulating cyclic ADP ribose), and enhances agonist-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) responses in human airway smooth muscle (ASM). We previously demonstrated that caveolae and their constituent protein caveolin-1 are important for ASM $[Ca^{2+}]_i$ regulation, which is further enhanced by TNF α . Whether caveolae and CD38 are functionally linked in mediating TNF α effects is unknown. In this regard, whether the related cavin proteins (cavin-1 and -3) that maintain structure and function of caveolae play a role is also not known. In the present study, we hypothesized that TNF α effects on CD38 expression and function in human ASM involve caveolae. Caveolar fractions from isolated human ASM cells expressed CD38 and its expression was upregulated by exposure to 20 ng/ml TNF α (48 h). ASM cells expressed cavin-1 and cavin-3, which were also upregulated by TNF α . Knockdown of caveolin-1, cavin-1 or cavin-3 (using siRNA) all significantly reduced CD38 expression and ADP-ribosyl cyclase activity in the presence or absence of TNF α . Furthermore, caveolin-1, cavin-1 and cavin-3 siRNAs reduced $[Ca^{2+}]_i$ responses to histamine under control conditions, and blunted the enhanced $[Ca^{2+}]_i$ responses in TNF α -exposed cells. These data demonstrate that CD38 is expressed within caveolae and its function is linked to the caveolar regulatory proteins caveolin-1, cavin-1 and -3. The link between caveolae and CD38 is further enhanced during airway inflammation demonstrating the important role of caveolae in regulation of $[Ca^{2+}]_i$ and contractility in the airway.

3.2123 **Dissecting Functions of the Conserved Oligomeric Golgi Tethering Complex Using a Cell-Free Assay**
Cottam, N.P., Wilson, K.M., Ng, B.G., Körner, C., Freeze, H.H. and Ungar, D.

Traffic, ***15(1)***, *12-21 (2014)*

Vesicle transport sorts proteins between compartments and is thereby responsible for generating the non-uniform protein distribution along the eukaryotic secretory and endocytic pathways. The mechanistic details of specific vesicle targeting are not yet well characterized at the molecular level. We have developed a cell-free assay that reconstitutes vesicle targeting utilizing the recycling of resident enzymes within the Golgi apparatus. The assay has physiological properties, and could be used to show that the two lobes of the conserved oligomeric Golgi tethering complex play antagonistic roles in *trans*-Golgi vesicle targeting. Moreover, we can show that the assay is sensitive to several different congenital defects that disrupt Golgi function and therefore cause glycosylation disorders. Consequently, this assay will allow mechanistic insight into the targeting step of vesicle transport at the Golgi, and could also be useful for characterizing some novel cases of congenital glycosylation disorders

3.2124 Activation of Src and release of intracellular calcium by phosphatidic acid during Xenopus laevis fertilization

Bates, R.C., Fees, C.P., Holland, W.L:, Winger, C.C., Batbayar, K., Ancar, R., Bergren, T., Petkoff, D. and Stich, B.J.
Developmental Biol., ***386***, *165-180 (2014)*

We report a new step in the fertilization in *Xenopus laevis* which has been found to involve activation of Src tyrosine kinase to stimulate phospholipase C-γ (PLC-γ) which increases inositol 1,4,5-trisphosphate (IP3) to release intracellular calcium ([Ca]*i*). Molecular species analysis and mass measurements suggested that sperm activate phospholipase D (PLD) to elevate phosphatidic acid (PA). We now report that PA mass increased 2.7 fold by 1 min after insemination and inhibition of PA production by two methods inhibited activation of Src and PLC γ, increased [Ca]*i* and other fertilization events. As compared to 14 other lipids, PA specifically bound *Xenopus* Src but not PLC γ. Addition of synthetic PA activated egg Src (an action requiring intact lipid rafts) and PLC γ as well as doubling the amount of PLC γ in rafts. In the absence of elevated [Ca]*i*, PA addition elevated IP3 mass to levels equivalent to that induced by sperm (but twice that achieved by calcium ionophore). Finally, PA induced [Ca]*i* release that was blocked by an IP3 receptor inhibitor. As only PLD1b message was detected, and Western blotting did not detect PLD2, we suggest that sperm activate PLD1b to elevate PA which then binds to and activates Src leading to PLC γ stimulation, IP3 elevation and [Ca]*i* release. Due to these and other studies, PA may also play a role in membrane fusion events such as sperm-egg fusion, cortical granule exocytosis, the elevation of phosphatidylinositol 4,5-bisphosphate and the large, late increase in sn 1,2-diacylglycerol in fertilization.

3.2125 Comprehensive proteomic profiling of outer membrane vesicles from Campylobacter jejuni

Jang, K-S., Sweredoski, M.J., Graham, R.L.J., Hess, S. and Clemons Jr. W.M.
J. Proteomics, ***98***, *90-98 (2014)*

Gram-negative bacteria constitutively release outer membrane vesicles (OMVs) during cell growth that play significant roles in bacterial survival, virulence and pathogenesis. In this study, comprehensive proteomic analysis of OMVs from a human gastrointestinal pathogen *Campylobacter jejuni* NCTC11168 was performed using high-resolution mass spectrometry. The OMVs of *C. jejuni* NCTC11168 were isolated from culture supernatants then characterized using electron microscopy and dynamic light scattering revealing spherical OMVs of an average diameter of 50 nm. We then identified 134 vesicular proteins using high-resolution LTQ-Orbitrap mass spectrometry. Subsequent functional analysis of the genes revealed the relationships of the vesicular proteins. Furthermore, known N-glycoproteins were identified from the list of the vesicular proteome, implying the potential role of the OMVs as a delivery means for biologically relevant bacterial glycoproteins. These results enabled us to elucidate the overall proteome profile of pathogenic bacterium *C. jejuni* and to speculate on the function of OMVs in bacterial infections and communication.

3.2126 CKIP-1 Is an Intrinsic Negative Regulator of T-Cell Activation through an Interaction with CARMA1

Sakamoto, T., Kobayashi, M., Tada, K., Shinohara, M., Io, K., Nagata, K., Iwai, F., Takiuchi, Y., Arai, Y., Yamahita, K., Shindo, K., Kadowaki, N., Koyanagi, Y. and Takaori-Kondo, A.
PloS One, ***9(1)***, *e85762 (2014)*

The transcription factor NF-κB plays a key regulatory role in lymphocyte activation and generation of immune response. Stimulation of T cell receptor (TCR) induces phosphorylation of CARMA1 by PKCθ, resulting in formation of CARMA1-Bcl10-MALT1 (CBM) complex at lipid rafts and subsequently leading to NF-κB activation. While many molecular events leading to NF-κB activation have been reported, it is less understood how this activation is negatively regulated. We performed a cell-based screening for negative regulators of TCR-mediated NF-κB activation, using mutagenesis and complementation cloning strategies. Here we show that casein kinase-2 interacting protein-1 (CKIP-1) suppresses PKCθ-CBM-NF-κB signaling. We found that CKIP-1 interacts with CARMA1 and competes with PKCθ for association. We further confirmed that a PH domain of CKIP-1 is required for association with CARMA1 and its inhibitory effect. CKIP-1 represses NF-κB activity in unstimulated cells, and inhibits NF-κB activation induced by stimulation with PMA or constitutively active PKCθ, but not by stimulation with TNFα. Interestingly, CKIP-1 does not inhibit NF-κB activation induced by CD3/CD28 costimulation, which caused dissociation of CKIP-1 from lipid rafts. These data suggest that CKIP-1 contributes maintenance of a resting state on NF-κB activity or prevents T cells from being activated by inadequate signaling. In conclusion, we demonstrate that CKIP-1 interacts with CARMA1 and has an inhibitory effect on PKCθ-CBM-NF-κB signaling.

3.2127 Acute Phencyclidine Treatment Induces Extensive and Distinct Protein Phosphorylation in Rat Frontal Cortex

Palmowski, P., Rogowska-Wrzesinska, A., Williamson, J., Beck, H.C., Mikkelsen, J.D., Hansen, H.H. and Jensen, O.N.

J. Proteome Res., ***13***, *1578-1592 (2014)*

Phencyclidine (PCP), a noncompetitive N-methyl-d-aspartate receptor antagonist, induces psychotomimetic effects in humans and animals. Administration of PCP to rodents is used as a preclinical model for schizophrenia; however, the molecular mechanisms underlying the symptoms remain largely unknown. Acute PCP treatment rapidly induces behavioral and cognitive deficits; therefore, post-translational regulation of protein activity is expected to play a role at early time points. We performed mass-spectrometry-driven quantitative analysis of rat frontal cortex 15, 30, or 240 min after the administration of PCP (10 mg/kg). We identified and quantified 23 548 peptides, including 4749 phosphopeptides, corresponding to 2604 proteins. A total of 352 proteins exhibited altered phosphorylation levels, indicating that protein phosphorylation is involved in the acute response to PCP. Computational assessment of the regulated proteins biological function revealed that PCP perturbs key processes in the frontal cortex including calcium homeostasis, organization of cytoskeleton, endo/exocytosis, and energy metabolism. This study on acute PCP treatment provides the largest proteomics and phosphoproteomics data sets to date of a preclinical model of schizophrenia. Our findings contribute to the understanding of alterations in glutamatergic neurotransmission in schizophrenia and provide a foundation for discovery of novel targets for pharmacological intervention.

3.2128 A Functional Interplay between the Small GTPase Rab11a and Mitochondria-shaping Proteins Regulates Mitochondrial Positioning and Polarization of the Actin Cytoskeleton Downstream of Src Family Kinases

Landry, M-C., Champagne, C., Boulanger, M-C., Jette, A., Fuchs, M., Dziengelwski, C. and Lavoie, J.N.

J. Biol. Chem., ***289(4)***, *2230-2249 (2014)*

It is believed that mitochondrial dynamics is coordinated with endosomal traffic rates during cytoskeletal remodeling, but the mechanisms involved are largely unknown. The adenovirus early region 4 ORF4 protein (E4orf4) subverts signaling by Src family kinases (SFK) to perturb cellular morphology, membrane traffic, and organellar dynamics and to trigger cell death. Using E4orf4 as a model, we uncovered a functional connection between mitochondria-shaping proteins and the small GTPase Rab11a, a key regulator of polarized transport via recycling endosomes. We found that E4orf4 induced dramatic changes in the morphology of mitochondria along with their mobilization at the vicinity of a polarized actin network typifying E4orf4 action, in a manner controlled by SFK and Rab11a. Mitochondrial remodeling was associated with increased proximity between Rab11a and mitochondrial membranes, changes in fusion-fission dynamics, and mitochondrial relocalization of the fission factor dynamin-related protein 1 (Drp1), which was regulated by the Rab11a effector protein FIP1/RCP. Knockdown of FIP1/RCP or inhibition of Drp1 markedly impaired mitochondrial remodeling and actin assembly, involving Rab11a-mediated mitochondrial dynamics in E4orf4-induced signaling. A similar mobilization of mitochondria near actin-rich structures was mediated by Rab11 and Drp1 in viral Src-transformed cells and contributed

to the biogenesis of podosome rosettes. These findings suggest a role for Rab11a in the trafficking of Drp1 to mitochondria upon SFK activation and unravel a novel functional interplay between Rab11a and mitochondria during reshaping of the cell cytoskeleton, which would facilitate mitochondria redistribution near energy-requiring actin-rich structures.

3.2129 TRPC6 participates in the regulation of cytosolic basal calcium concentration in murine resting platelets

Albarran, L., Berna-Erro, A., Dionisio, N., Redondo, P.C., Lopez, E., Lopez, J.J., Salido, G.M., Sabate, J.M.B. and Rasado, J.A.
*Biochim. Biophys. Acta, **1843**, 789-796 (2014)*

Cytosolic-free Ca^{2+} plays a crucial role in blood platelet function and is essential for thrombosis and hemostasis. Therefore, cytosolic-free Ca^{2+} concentration is tightly regulated in this cell. TRPC6 is expressed in platelets, and an important role for this Ca^{2+} channel in Ca^{2+} homeostasis has been reported in other cell types. The aim of this work is to study the function of TRPC6 in platelet Ca^{2+} homeostasis. The absence of TRPC6 resulted in an 18.73% decreased basal $[Ca^{2+}]_c$ in resting platelets as compared to control cells. Further analysis confirmed a similar Ca^{2+} accumulation in wild-type and TRPC6-deficient mice; however, passive Ca^{2+} leak rates from agonist-sensitive intracellular stores were significantly decreased in TRPC6-deficient platelets. Biotinylation studies indicated the presence of an intracellular TRPC6 population, and subcellular fractionation indicated their presence on endoplasmic reticulum membranes. Moreover, the presence of intracellular calcium release in platelets stimulated with 1-oleoyl-2-acetyl-sn-glycerol further suggested a functional TRPC6 population located on the intracellular membranes surrounding calcium stores. However, coimmunoprecipitation assay confirmed the absence of STIM1-TRPC6 interactions in resting conditions. This findings together with the absence of extracellular Mn^{2+} entry in resting wild-type platelets indicate that the plasma membrane TRPC6 fraction does not play a significant role in the maintenance of basal $[Ca^{2+}]_c$ in mouse platelets. Our results suggest an active participation of the intracellular TRPC6 fraction as a regulator of basal $[Ca^{2+}]_c$, controlling the passive Ca^{2+} leak rate from agonist-sensitive intracellular Ca^{2+} stores in resting platelets.

3.2130 The endoplasmic reticulum-mitochondria connection: One touch, multiple functions

Marchi, S., Patergnani, S. and Pinton, P.
*Biochim. Biophys. Acta, **1837**, 461-469 (2014)*

The endoplasmic reticulum (ER) and mitochondria are tubular organelles with a characteristic "network structure" that facilitates the formation of interorganellar connections. The ER and mitochondria join together at multiple contact sites to form specific domains, termed mitochondria-ER associated membranes (MAMs), with distinct biochemical properties and a characteristic set of proteins. The functions of these two organelles are coordinated and executed at the ER-mitochondria interface, which provides a platform for the regulation of different processes. The roles played by the ER-mitochondria interface range from the coordination of calcium transfer to the regulation of mitochondrial fission and inflammasome formation as well as the provision of membranes for autophagy. The novel and unconventional processes that occur at the ER-mitochondria interface demonstrate its multifunctional and intrinsically dynamic nature. This article is part of a Special Issue entitled: Dynamic and ultrastructure of bioenergetic membranes and their components.

3.2131 CIB1 Synergizes with EphrinA2 to Regulate Kaposi's Sarcoma-Associated Herpesvirus Macropinocytic Entry in Human Microvascular Dermal Endothelial Cells

Bandyopadhyay, C., Valiya-Veettil, M., Dutta, D., Chakraborty, S. and Chandran, B.
*PloS Pathogens, **10(2)**, e1003941 (2014)*

KSHV envelope glycoproteins interact with cell surface heparan sulfate and integrins, and activate FAK, Src, PI3-K, c-Cbl, and Rho-GTPase signal molecules in human microvascular dermal endothelial (HMVEC-d) cells. c-Cbl mediates the translocation of virus bound α3β1 and αVβ3 integrins into lipid rafts (LRs), where KSHV interacts and activates EphrinA2 (EphA2). EphA2 associates with c-Cbl-myosin IIA and augmented KSHV-induced Src and PI3-K signals in LRs, leading to bleb formation and macropinocytosis of KSHV. To identify the factor(s) coordinating the EphA2-signal complex, the role of CIB1 (calcium and integrin binding protein-1) associated with integrin signaling was analyzed. CIB1 knockdown did not affect KSHV binding to HMVEC-d cells but significantly reduced its entry and gene expression. In contrast, CIB1 overexpression increased KSHV entry in 293 cells. Single virus particle

infection and trafficking during HMVEC-d cell entry was examined by utilizing DiI (envelope) and BrdU (viral DNA) labeled virus. CIB1 was associated with KSHV in membrane blebs and in Rab5 positive macropinocytic vesicles. CIB1 knockdown abrogated virus induced blebs, macropinocytosis and virus association with the Rab5 macropinosome. Infection increased the association of CIB1 with LRs, and CIB1 was associated with EphA2 and KSHV entry associated signal molecules such as Src, PI3-K, and c-Cbl. CIB1 knockdown significantly reduced the infection induced EphA2, Src and Erk1/2 activation. Mass spectrometry revealed the simultaneous association of CIB1 and EphA2 with the actin cytoskeleton modulating myosin IIA and alpha-actinin 4 molecules, and CIB1 knockdown reduced EphA2's association with myosin IIA and alpha-actinin 4. Collectively, these studies revealed for the first time that CIB1 plays a role in virus entry and macropinocytosis, and suggested that KSHV utilizes CIB1 as one of the key molecule(s) to coordinate and sustain the EphA2 mediated signaling involved in its entry, and CIB1 is an attractive therapeutic target to block KSHV infection.

3.2132 A paradigm shift for extracellular vesicles as small RNA carriers: from cellular waste elimination to therapeutic applications
Hagiwara, K., Ochiya, T. and Kosaka, N.
*Drug Deliv.and Transl. Res., **4**:31 (2014)*

RNA interference (RNAi) is an important avenue for target-specific gene silencing that is mainly performed by either small interfering RNAs (siRNAs) or microRNAs (miRNAs). This novel method is rapidly becoming a powerful tool for gene therapy. However, the rapid degradation of siRNAs and miRNAs and the limited duration of their action in vivo call for an efficient delivery technology. Recently, increasing attention has been paid to the use of extracellular vesicles (EVs) as delivery systems. The use of EVs as small RNA carriers has multiple advantages over conventional delivery systems. In this review, we summarize recent findings regarding the potential application of EVs as small RNA delivery systems. Moreover, we focus on some of the obstacles to EV-based therapeutics.

3.2133 Eucommia ulmoides Cortex, Geniposide and Aucubin Regulate Lipotoxicity through the Inhibition of Lysosomal BAX
Lee, G-H., Lee, M-R., Lee, H-Y., Kim, S.H., Kim, H-K., Kim, H-R. and Chae, H-J.
*PloS One, **9(2)**, e88017 (2014)*

In this study we examined the inhibition of hepatic dyslipidemia by *Eucommia ulmoides* extract (EUE). Using a screening assay for BAX inhibition we determined that EUE regulates BAX-induced cell death. Among various cell death stimuli tested EUE regulated palmitate-induced cell death, which involves lysosomal BAX translocation. EUE rescued palmitate-induced inhibition of lysosomal V-ATPase, α-galactosidase, α-mannosidase, and acid phosphatase, and this effect was reversed by bafilomycin, a lysosomal V-ATPase inhibitor. The active components of EUE, aucubin and geniposide, showed similar inhibition of palmitate-induced cell death to that of EUE through enhancement of lysosome activity. Consistent with these in vitro findings, EUE inhibited the dyslipidemic condition in a high-fat diet animal model by regulating the lysosomal localization of BAX. This study demonstrates that EUE regulates lipotoxicity through a novel mechanism of enhanced lysosomal activity leading to the regulation of lysosomal BAX activation and cell death. Our findings further indicate that geniposide and aucubin, active components of EUE, may be therapeutic candidates for non-alcoholic fatty liver disease.

3.2134 Role for Mycobacterium tuberculosis Membrane Vesicles in Iron Acquisition
Prados-Rosales, R., Weinrick, B.C., Pique, D.G., Jacobs Jr, W.R., Casadevall, A. and rodriguez, G.M.
*J. Bacteriol., **196(6)**, 1250-1256 (2014)*

Mycobacterium tuberculosis releases membrane vesicles packed with molecules that can modulate the immune response. Because environmental conditions often influence the production and content of bacterial vesicles, this study examined M. tuberculosis microvesicles released under iron limitation, a common condition faced by pathogens inside the host. The findings indicate that M. tuberculosis increases microvesicle production in response to iron restriction and that these microvesicles contain mycobactin, which can serve as an iron donor and supports replication of iron-starved mycobacteria. Consequently, the results revealed a role of microvesicles in iron acquisition in M. tuberculosis, which can be critical for survival in the host.

3.2135 **On the Formation of Lipid Droplets in Human Adipocytes: The Organization of the Perilipin–Vimentin Cortex**
Heid, H., Rickelt, S., Zimbelmann, R., Winter, S., Schumacher, H., Dörflinger, Y., Kuhn, C and Franke, W.W.
*PloS One, **9(2)**, e90386 (2014)*

We report on the heterogeneity and diversity of lipid droplets (LDs) in early stages of adipogenesis by elucidating the cell and molecular biology of amphiphilic and cytoskeletal proteins regulating and stabilizing the generation of LDs in human adipose cells. A plethora of distinct and differently sized LDs was detected by a brief application of adipocyte differentiation medium and additional short treatment with oleic acid. Using these cells and highly specific antibodies for LD-binding proteins of the perilipin (PLIN) family, we could distinguish between endogenously derived LDs (endogenous LDs) positive for perilipin from exogenously induced LDs (exogenous LDs) positive for adipophilin, TIP47 and S3-12. Having optimized these stimulation conditions, we used early adipogenic differentiation stages to investigate small-sized LDs and concentrated on LD-protein associations with the intermediate-sized filament (IF) vimentin. This IF protein was described earlier to surround lipid globules, showing spherical, cage-like structures. Consequently - by biochemical methods, by immunofluorescence microscopy and by electron- and immunoelectron microscopy - various stages of emerging lipid globules were revealed with perilipin as linking protein between LDs and vimentin. For this LD-PLIN-Vimentin connection, a model is now proposed, suggesting an interaction of proteins via opposed charged amino acid domains respectively. In addition, multiple sheaths of smooth endoplasmic reticulum cisternae surrounding concentrically nascent LDs are shown. Based on our comprehensive localization studies we present and discuss a novel pathway for the LD formation.

3.2136 **Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast coq null mutants**
He, C.H., Xie, L.X., Allan, C.M., Tran, U.C.and Clarke, C.F.
*Biochim. Biophys. Acta, **1841**, 630-644 (2014)*

Coenzyme Q biosynthesis in yeast requires a multi-subunit Coq polypeptide complex. Deletion of any one of the *COQ* genes leads to respiratory deficiency and decreased levels of the Coq4, Coq6, Coq7, and Coq9 polypeptides, suggesting that their association in a high molecular mass complex is required for stability. Over-expression of the putative Coq8 kinase in certain *coq* null mutants restores steady-state levels of the sensitive Coq polypeptides and promotes the synthesis of late-stage Q-intermediates. Here we show that over-expression of Coq8 in yeast *coq* null mutants profoundly affects the association of several of the Coq polypeptides in high molecular mass complexes, as assayed by separation of digitonin extracts of mitochondria by two-dimensional blue-native/SDS PAGE. The Coq4 polypeptide persists at high molecular mass with over-expression of Coq8 in *coq3*, *coq5*, *coq6*, *coq7*, *coq9*, and *coq10* mutants, indicating that Coq4 is a central organizer of the Coq complex. Supplementation with exogenous Q_6 increased the steady-state levels of Coq4, Coq7, and Coq9, and several other mitochondrial polypeptides in select *coq* null mutants, and also promoted the formation of late-stage Q-intermediates. Q supplementation may stabilize this complex by interacting with one or more of the Coq polypeptides. The stabilizing effects of exogenously added Q_6 or over-expression of Coq8 depend on Coq1 and Coq2 production of a polyisoprenyl intermediate. Based on the observed interdependence of the Coq polypeptides, the effect of exogenous Q_6, and the requirement for an endogenously produced polyisoprenyl intermediate, we propose a new model for the Q-biosynthetic complex, termed the CoQ-synthome.

3.2137 **Selective Association of Outer Surface Lipoproteins with the Lipid Rafts of Borrelia burgdorferi**
Toledo, A., Crowley, J.T., Coleman, J.L. et al
*mBio, **5(2)**, e00899 (2014)*

Borrelia burgdorferi contains unique cholesterol-glycolipid-rich lipid rafts that are associated with lipoproteins. These complexes suggest the existence of macromolecular structures that have not been reported for prokaryotes. Outer surface lipoproteins OspA, OspB, and OspC were studied for their participation in the formation of lipid rafts. Single-gene deletion mutants with deletions of Δ*ospA*, Δ*ospB*, and Δ*ospC* and a spontaneous gene mutant, strain B313, which does not express OspA and OspB, were used to establish their structural roles in the lipid rafts. All mutant strains used in this study produced detergent-resistant membranes, a common characteristic of lipid rafts, and had similar lipid and protein slot blot profiles. Lipoproteins OspA and OspB but not OspC were shown to be associated with lipid rafts by transmission electron microscopy. When the ability to form lipid rafts in live *B. burgdorferi* spirochetes

was measured by fluorescence resonance energy transfer (FRET), strain B313 showed a statistically significant lower level of segregation into ordered and disordered membrane domains than did the wild-type and the other single-deletion mutants. The transformation of a B313 strain with a shuttle plasmid containing *ospA* restored the phenotype shared by the wild type and the single-deletion mutants, demonstrating that OspA and OspB have redundant functions. In contrast, a transformed B313 overexpressing OspC neither rescued the FRET nor colocalized with the lipid rafts. Because these lipoproteins are expressed at different stages of the life cycle of *B. burgdorferi*, their selective association is likely to have an important role in the structure of prokaryotic lipid rafts and in the organism's adaptation to changing environments.

3.2138 Proteomic Profiling of Autophagosome Cargo in Saccharomyces cerevisiae
Suzuki, K., Nakamura, S., Morimoto, M., Fujii, K., Noda, N.N., Inagaki, F. and Ohsumi, Y.
*Plos One, **9(3)**, e91651 (2014)*

Macroautophagy (autophagy) is a bulk protein-degradation system ubiquitously conserved in eukaryotic cells. During autophagy, cytoplasmic components are enclosed in a membrane compartment, called an autophagosome. The autophagosome fuses with the vacuole/lysosome and is degraded together with its cargo. Because autophagy is important for the maintenance of cellular homeostasis by degrading unwanted proteins and organelles, identification of autophagosome cargo proteins (i.e., the targets of autophagy) will aid in understanding the physiological roles of autophagy. In this study, we developed a method for monitoring intact autophagosomes *ex vivo* by detecting the fluorescence of GFP-fused aminopeptidase I, the best-characterized selective cargo of autophagosomes in *Saccharomyces cerevisiae*. This method facilitated optimization of a biochemical procedure to fractionate autophagosomes. A combination of LC-MS/MS with subsequent statistical analyses revealed a list of autophagosome cargo proteins; some of these are selectively enclosed in autophagosomes and delivered to the vacuole in an Atg11-independent manner. The methods we describe will be useful for analyzing the mechanisms and physiological significance of Atg11-independent selective autophagy.

3.2139 GPx8 peroxidase prevents leakage of H2O2 from the endoplasmic reticulum
Ramming, T., Hansen, H.G., Nagata, K., Ellgaard, L. and Appenzeller-Herzog, C.
*Free Radical Biology and Medicine, **70**, 106-116 (2014)*

Unbalanced endoplasmic reticulum (ER) homeostasis (ER stress) leads to increased generation of reactive oxygen species (ROS). Disulfide-bond formation in the ER by Ero1 family oxidases produces hydrogen peroxide (H_2O_2) and thereby constitutes one potential source of ER-stress-induced ROS. However, we demonstrate that Ero1 α -derived H_2O_2 is rapidly cleared by glutathione peroxidase (GPx) 8. In 293 cells, GPx8 and reduced/activated forms of Ero1 α co-reside in the rough ER subdomain. Loss of GPx8 causes ER stress, leakage of Ero1 α -derived H_2O_2 to the cytosol, and cell death. In contrast, peroxiredoxin (Prx) IV, another H_2O_2-detoxifying rough ER enzyme, does not protect from Ero1 α -mediated toxicity, as is currently proposed. Only when Ero1 α -catalyzed H_2O_2 production is artificially maximized can PrxIV participate in its reduction. We conclude that the peroxidase activity of the described Ero1 α -GPx8 complex prevents diffusion of Ero1 α -derived H_2O_2 within and out of the rough ER. Along with the induction of *GPX8* in ER-stressed cells, these findings question a ubiquitous role of Ero1 α as a producer of cytoplasmic ROS under ER stress.

3.2140 Protein Kinase C Mediates Enterohemorrhagic Escherichia coli O157:H7-Induced Attaching and Effacing Lesions
Shen-Tu, G., Kim, H., Liu, M., Johnson-Henry, C. and Sherman, P.M.
*Infect. Immun., **82(4)**, 1648-1656 (2014)*

Enterohemorrhagic Escherichia coli serotype O157:H7 causes outbreaks of diarrhea, hemorrhagic colitis, and the hemolytic-uremic syndrome. E. coli O157:H7 intimately attaches to epithelial cells, effaces microvilli, and recruits F-actin into pedestals to form attaching and effacing lesions. Lipid rafts serve as signal transduction platforms that mediate microbe-host interactions. The aims of this study were to determine if protein kinase C (PKC) is recruited to lipid rafts in response to E. coli O157:H7 infection and what role it plays in attaching and effacing lesion formation. HEp-2 and intestine 407 tissue culture epithelial cells were challenged with E. coli O157:H7, and cell protein extracts were then separated by buoyant density ultracentrifugation to isolate lipid rafts. Immunoblotting for PKC was performed, and localization in lipid rafts was confirmed with an anti-caveolin-1 antibody. Isoform-specific PKC small

interfering RNA (siRNA) was used to determine the role of PKC in E. coli O157:H7-induced attaching and effacing lesions. In contrast to uninfected cells, PKC was recruited to lipid rafts in response to E. coli O157:H7. Metabolically active bacteria and cells with intact lipid rafts were necessary for the recruitment of PKC. PKC recruitment was independent of the intimin gene, type III secretion system, and the production of Shiga toxins. Inhibition studies, using myristoylated PKCζ pseudosubstrate, revealed that atypical PKC isoforms were activated in response to the pathogen. Pretreating cells with isoform-specific PKC siRNA showed that PKCζ plays a role in E. coli O157:H7-induced attaching and effacing lesions. We concluded that lipid rafts mediate atypical PKC signal transduction responses to E. coli O157:H7. These findings contribute further to the understanding of the complex array of microbe-eukaryotic cell interactions that occur in response to infection.

3.2141 Hijacking of RIG-I Signaling Proteins into Virus-Induced Cytoplasmic Structures Correlates with the Inhibition of Type I Interferon Responses
Santiago, F.W., Covaleda, L.M., Sanchez-Aparicio, M.T., Silvas, J.A., Diaz-Vizarreta, A.C., patel, J.R., Popov, V., Yu, X-j., Garcia-Sastre, A. and Aguilar, P.V.
J. Virol., ***88(8)***, *4572-4585 (2014)*

Recognition of viral pathogens by the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family results in the activation of type I interferon (IFN) responses. To avoid this response, most viruses have evolved strategies that target different essential steps in the activation of host innate immunity. In this study, we report that the nonstructural protein NSs of the newly described severe fever with thrombocytopenia syndrome virus (SFTSV) is a potent inhibitor of IFN responses. The SFTSV NSs protein was found to inhibit the activation of the beta interferon (IFN-β) promoter induced by viral infection and by a RIG-I ligand. Astonishingly, we found that SFTSV NSs interacts with and relocalizes RIG-I, the E3 ubiquitin ligase TRIM25, and TANK-binding kinase 1 (TBK1) into SFTSV NSs-induced cytoplasmic structures. Interestingly, formation of these SFTSV NSs-induced structures occurred in the absence of the Atg7 gene, a gene essential for autophagy. Furthermore, confocal microscopy studies revealed that these SFTSV NSs-induced structures colocalize with Rab5 but not with Golgi apparatus or endoplasmic reticulum markers. Altogether, the data suggest that sequestration of RIG-I signaling molecules into endosome-like structures may be the mechanism used by SFTSV to inhibit IFN responses and point toward a novel mechanism for the suppression of IFN responses.

3.2142 Improved Functional Expression of Human Cardiac Kv1.5 Channels and Trafficking-Defective Mutants by Low Temperature Treatment
Ding, W-G., Xie, Y., Toyoda, F. and Matsuura, H.
Plos One, ***9(3)***, *e92923 (2014)*

We herein investigated the effect of low temperature exposure on the expression, degradation, localization and activity of human Kv1.5 (hKv1.5). In hKv1.5-expressing CHO cells, the currents were significantly increased when cultured at a reduced temperature (28°C) compared to those observed at 37°C. Western blot analysis indicated that the protein levels (both immature and mature proteins) of hKv1.5 were significantly elevated under the hypothermic condition. Treatment with a proteasome inhibitor, MG132, significantly increased the immature, but not the mature, hKv1.5 protein at 37°C, however, there were no changes in either the immature or mature hKv1.5 proteins at low temperature following MG132 exposure. These observations suggest that the enhancement of the mature hKv1.5 protein at reduced temperature may not result from the inhibition of proteolysis. Moreover, the hKv1.5 fluorescence signal in the cells increased significantly on the cell surface at 28°C versus those cultured at 37°C. Importantly, the low temperature treatment markedly shifted the subcellular distribution of the mature hKv1.5, which showed considerable overlap with the trans-Golgi component. Experiments using tunicamycin, an inhibitor of N-glycosylation, indicated that the N-glycosylation of hKv1.5 is more effective at 28°C than at 37°C. Finally, the hypothermic treatment also rescued the protein expression and currents of trafficking-defective hKv1.5 mutants. These results indicate that low temperature exposure stabilizes the protein in the cellular organelles or on the plasma membrane, and modulates its maturation and trafficking, thus enhancing the currents of hKv1.5 and its trafficking defect mutants.

3.2143 Herp coordinates compartmentalization and recruitment of HRD1 and misfolded proteins for ERAD
Leitman, J., Shenkman, M., Gofman, Y., Shtern, O. N., Ben-Tal, N., Hendershot, L.M. and lederkremer, G.Z.
Mol. Biol. Cell, ***25***, *1050-1060 (2014)*

A functional unfolded protein response (UPR) is essential for endoplasmic reticulum (ER)-associated degradation (ERAD) of misfolded secretory proteins, reflecting the fact that some level of UPR activation must exist under normal physiological conditions. A coordinator of the UPR and ERAD processes has long been sought. We previously showed that the PKR-like, ER-localized eukaryotic translation initiation factor 2α kinase branch of the UPR is required for the recruitment of misfolded proteins and the ubiquitin ligase HRD1 to the ER-derived quality control compartment (ERQC), a staging ground for ERAD. Here we show that homocysteine-induced ER protein (Herp), a protein highly upregulated by this UPR branch, is responsible for this compartmentalization. Herp localizes to the ERQC, and our results suggest that it recruits HRD1, which targets to ERAD the substrate presented by the OS-9 lectin at the ERQC. Predicted overall structural similarity of Herp to the ubiquitin-proteasome shuttle hHR23, but including a transmembrane hairpin, suggests that Herp may function as a hub for membrane association of ERAD machinery components, a key organizer of the ERAD complex.

3.2144 Ubiquitin-like (UBX)-domain-containing protein, UBXN2A, promotes cell death by interfering with the p53-Mortalin interactions in colon cancer cells

Sane, S., Abdullah, A., Boudreau, D.A., Autenried, R.K., Gupta, B.K., Wang, X., Wang, H., Schlenker, E.H., Zhang, D., Telleria, C., Huang, L., Chauhan, S.C. and Rezvani, K.
*Cell Death and Disease, **5**, e1118 (2014)*

Mortalin (mot-2) induces inactivation of the tumor suppressor p53's transcriptional and apoptotic functions by cytoplasmic sequestration of p53 in select cancers. The mot-2-dependent cytoprotective function enables cancer cells to support malignant transformation. Abrogating the p53-mot-2 interaction can control or slow down the growth of cancer cells. In this study, we report the discovery of a ubiquitin-like (UBX)-domain-containing protein, UBXN2A, which binds to mot-2 and consequently inhibits the binding between mot-2 and p53. Genetic analysis showed that UBXN2A binds to mot-2's substrate binding domain, and it partly overlaps p53's binding site indicating UBXN2A and p53 likely bind to mot-2 competitively. By binding to mot-2, UBXN2A releases p53 from cytosolic sequestration, rescuing the tumor suppressor functions of p53. Biochemical analysis and functional assays showed that the overexpression of UBXN2A and the functional consequences of unsequestered p53 trigger p53-dependent apoptosis. Cells expressing shRNA against UBXN2A showed the opposite effect of that seen with UBXN2A overexpression. The expression of UBXN2A and its apoptotic effects were not observed in normal colonic epithelial cells and p53−/− colon cancer cells. Finally, significant reduction in tumor volume in a xenograft mouse model in response to UBXN2A expression was verified *in vivo*. Our results introduce UBXN2A as a home defense response protein, which can reconstitute inactive p53-dependent apoptotic pathways. Inhibition of mot-2-p53 interaction by UBXN2A is an attractive therapeutic strategy in mot-2-elevated tumors.

3.2145 Mutation of SLC35D3 Causes Metabolic Syndrome by Impairing Dopamine Signaling in Striatal D1 Neurons

Zhang, Z., Hao, C-J., Li, C-G., Zang, D-J., Zhao, J., Li, X-N., Wei, A-H., Wei, Z-B., Yang, L., He, X., Zhen, X-C., Gao, X., Speakman, J.R. and Li, W.
*PloS Genetics, **10(2)**, e1004124 (2014)*

Obesity is one of the largest health problems facing the world today. Although twin and family studies suggest about two-thirds of obesity is caused by genetic factors, only a small fraction of this variance has been unraveled. There are still large numbers of genes to be identified that cause variations in body fatness and the associated diseases encompassed in the metabolic syndrome (MetS). A locus near a sequence tagged site (STS) marker D6S1009 has been linked to obesity or body mass index (BMI). However, its genetic entity is unknown. D6S1009 is located in the intergenic region between *SLC35D3* and *NHEG1*. Here we report that the *ros* mutant mice harboring a recessive mutation in the *Slc35d3* gene show obesity and MetS and reduced membrane dopamine receptor D1 (D1R) with impaired dopamine signaling in striatal neurons. SLC35D3 is localized to both endoplasmic reticulum (ER) and early endosomes and interacts with D1R. In *ros* striatal D1 neurons, lack of SLC35D3 causes the accumulation of D1R on the ER to impair its ER exit. The MetS phenotype is reversible by the administration of D1R agonist to the *ros* mutant. In addition, we identified two mutations in the *SLC35D3* gene in patients with MetS, which alter the subcellular localization of SLC35D3. Our results suggest that the *SLC35D3* gene, close to the D6S1009 locus, is a candidate gene for MetS, which is involved in metabolic control in the central nervous system by regulating dopamine signaling.

3.2146 Human cytomegalovirus pUL37x1-induced calcium flux activates PKCα, inducing altered cell shape and accumulation of cytoplasmic vesicles
Sharon-Friling, R. and Shenk, T.
*PNAS, **111**, E1140-E1148 (2014)*

The human cytomegalovirus immediate-early protein pUL37x1 induces the release of Ca^{2+} stores from the endoplasmic reticulum into the cytosol. This release causes reorganization of the cellular actin cytoskeleton with concomitant cell rounding. Here we demonstrate that pUL37x1 activates Ca^{2+}-dependent protein kinase Cα (PKCα). Both PKCα and Rho-associated protein kinases are required for actin reorganization and cell rounding; however, only PKCα is required for the efficient production of virus progeny, arguing that HCMV depends on the kinase for a second function. PKCα activation is also needed for the production of large (1–5 μm) cytoplasmic vesicles late after infection. The production of these vesicles is blocked by inhibition of fatty acid or phosphatidylinositol-3-phosphate biosynthesis, and the failure to produce vesicles is correlated with substantially reduced production of enveloped virus capsids. These results connect earlier work identifying a requirement for lipid synthesis with specific morphological changes, and support the argument that the PKCα-induced large vesicles are either required for the efficient production of mature virus particles or serve as a marker for the process.

3.2147 RAB26 coordinates lysosome traffic and mitochondrial localization
Jin, R.U. and Mills, J.C.
*J. Cell Sci., **127**, 1018-1032 (2014)*

As they mature, professional secretory cells like pancreatic acinar and gastric chief cells induce the transcription factor MIST1 (also known as BHLHA15) to substantially scale up production of large secretory granules in a process that involves expansion of apical cytoplasm and redistribution of lysosomes and mitochondria. How a scaling factor like MIST1 rearranges cellular architecture simply by regulating expression levels of its transcriptional targets is unknown. RAB26 is a MIST1 target whose role in MIST1-mediated secretory cell maturation is also unknown. Here, we confirm that RAB26 expression, unlike most Rabs which are ubiquitously expressed, is tissue specific and largely confined to MIST1-expressing secretory tissues. Surprisingly, functional studies showed that RAB26 predominantly associated with LAMP1/cathepsin D lysosomes and not directly with secretory granules. Moreover, increasing RAB26 expression – by inducing differentiation of zymogen-secreting cells or by direct transfection – caused lysosomes to coalesce in a central, perinuclear region. Lysosome clustering in turn caused redistribution of mitochondria into distinct subcellular neighborhoods. The data elucidate a novel function for RAB26 and suggest a mechanism for how cells could increase transcription of key effectors to reorganize subcellular compartments during differentiation.

3.2148 Phospholipid flippase ATP8A2 is required for normal visual and auditory function and photoreceptor and spiral ganglion cell survival
Coleman, J.A., Zhu, X., Djajadi, H.R., Molday, L.L., Smith, R.S., Libby, R.T., John, S.W.M. and Molday, R.S.
*J. Cell Sci., **127**, 1138-1149 (2014)*

ATP8A2 is a P_4-ATPase that is highly expressed in the retina, brain, spinal cord and testes. In the retina, ATP8A2 is localized in photoreceptors where it uses ATP to transport phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the exoplasmic to the cytoplasmic leaflet of membranes. Although mutations in *ATP8A2* have been reported to cause mental retardation in humans and degeneration of spinal motor neurons in mice, the role of ATP8A2 in sensory systems has not been investigated. We have analyzed the retina and cochlea of ATP8A2-deficient mice to determine the role of ATP8A2 in visual and auditory systems. ATP8A2-deficient mice have shortened photoreceptor outer segments, a reduction in photoresponses and decreased photoreceptor viability. The ultrastructure and phagocytosis of the photoreceptor outer segment appeared normal, but the PS and PE compositions were altered and the rhodopsin content was decreased. The auditory brainstem response threshold was significantly higher and degeneration of spiral ganglion cells was apparent. Our studies indicate that ATP8A2 plays a crucial role in photoreceptor and spiral ganglion cell function and survival by maintaining phospholipid composition and contributing to vesicle trafficking.

3.2149 Slit Diaphragm Protein Neph1 and Its Signaling: A NOVEL THERAPEUTIC TARGET FOR PROTECTION OF PODOCYTES AGAINST GLOMERULAR INJURY

Arif, E., Rathore, Y.S., Kumari, b., Ashish, F., Wong, H.N., Holzman, L.B. and Nihalani, D.
J. Biol. Chem., ***289(14)***, *9502-9518 (2014)*

Podocytes are specialized epithelial cells that are critical components of the glomerular filtration barrier, and their dysfunction leads to proteinuria and renal failure. Therefore, preserving podocyte function is therapeutically significant. In this study, we identified Neph1 signaling as a therapeutic target that upon inhibition prevented podocyte damage from a glomerular injury-inducing agent puromycin aminonucleoside (PAN). To specifically inhibit Neph1 signaling, we used a protein transduction approach, where the cytoplasmic domain of Neph1 (Neph1CD) tagged with a protein transduction domain trans-activator of transcription was transduced in cultured podocytes prior to treatment with PAN. The PAN-induced Neph1 phosphorylation was significantly reduced in Neph1CD-transduced cells; in addition, these cells were resistant to PAN-induced cytoskeletal damage. The biochemical analysis using subfractionation studies showed that unlike control cells Neph1 was retained in the lipid raft fractions in the transduced cells following treatment with PAN, indicating that transduction of Neph1CD in podocytes prevented PAN-induced mislocalization of Neph1. In accordance, the immunofluorescence analysis further suggested that Neph1CD-transduced cells had increased ability to retain endogenous Neph1 at the membrane in response to PAN-induced injury. Similar results were obtained when angiotensin was used as an injury-inducing agent. Consistent with these observations, maintaining high levels of Neph1 at the membrane using a podocyte cell line overexpressing chimeric Neph1 increased the ability of podocytes to resist PAN-induced injury and PAN-induced albumin leakage. Using a zebrafish *in vivo* PAN and adriamycin injury models, we further demonstrated the ability of transduced Neph1CD to preserve glomerular function. Collectively, these results support the conclusion that inhibiting Neph1 signaling is therapeutically significant in preventing podocyte damage from glomerular injury.

3.2150 Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids

Khalifeh-Soltani, A., McKleroy,W., Sakuma, S., Cheung, Y.Y., Tharp, K., Qiu, Y., Turner, S.M., Chawla, A., Stahl, A. and Atabai, K.
Nature Med., ***20(2)***, *175-183 (2014)*

Fatty acids are integral mediators of energy storage, membrane formation and cell signaling. The pathways that orchestrate uptake of fatty acids remain incompletely understood. Expression of the integrin ligand Mfge8 is increased in human obesity and in mice on a high-fat diet, but its role in obesity is unknown. We show here that Mfge8 promotes the absorption of dietary triglycerides and the cellular uptake of fatty acid and that Mfge8-deficient ($Mfge8^{-/-}$) mice are protected from diet-induced obesity, steatohepatitis and insulin resistance. Mechanistically, we found that Mfge8 coordinates fatty acid uptake through $\alpha_v\beta_3$ integrin– and $\alpha_v\beta_5$ integrin–dependent phosphorylation of Akt by phosphatidylinositide-3 kinase and mTOR complex 2, leading to translocation of Cd36 and Fatp1 from cytoplasmic vesicles to the cell surface. Collectively, our results imply a role for Mfge8 in regulating the absorption and storage of dietary fats, as well as in the development of obesity and its complications.

3.2151 Proteomic profiling of cardiac tissue by isolation of nuclei tagged in specific cell types (INTACT)

Amin, N.M., Greco, T.M., Kuchenbrod, L.M., Rigney, M.M., Chung, M-i., Wallingfor, J.B., Cristea, I.M. and Conion, F.L.
Development, ***141***, *962-973 (2014)*

The proper dissection of the molecular mechanisms governing the specification and differentiation of specific cell types requires isolation of pure cell populations from heterogeneous tissues and whole organisms. Here, we describe a method for purification of nuclei from defined cell or tissue types in vertebrate embryos using INTACT (isolation of nuclei tagged in specific cell types). This method, previously developed in plants, flies and worms, utilizes *in vivo* tagging of the nuclear envelope with biotin and the subsequent affinity purification of the labeled nuclei. In this study we successfully purified nuclei of cardiac and skeletal muscle from *Xenopus* using this strategy. We went on to demonstrate the utility of this approach by coupling the INTACT approach with liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomic methodologies to profile proteins expressed in the nuclei of developing hearts. From these studies we have identified the *Xenopus* orthologs of 12 human proteins encoded by genes, which when mutated in human lead to congenital heart disease. Thus, by combining these technologies we are able to identify tissue-specific proteins that are expressed and required for normal vertebrate organ development.

3.2152 **The FTLD risk factor TMEM106B and MAP6 control dendritic trafficking of lysosomes**
Schwenk, B.M., Lang, C.M., Hogl, S., Tahirovic, S., Orozco, D., Rentzsch, K., Lichtenthaler, S.F., Hoogenraad, C.C., capell, A., Haass, C. and edhauer, D.
EMBO J., ***33(5)***, *450-467 (2014)*

TMEM106B is a major risk factor for frontotemporal lobar degeneration with TDP-43 pathology. TMEM106B localizes to lysosomes, but its function remains unclear. We show that TMEM106B knockdown in primary neurons affects lysosomal trafficking and blunts dendritic arborization. We identify microtubule-associated protein 6 (MAP6) as novel interacting protein for TMEM106B. MAP6 over-expression inhibits dendritic branching similar to TMEM106B knockdown. MAP6 knockdown fully rescues the dendritic phenotype of TMEM106B knockdown, supporting a functional interaction between TMEM106B and MAP6. Live imaging reveals that TMEM106B knockdown and MAP6 overexpression strongly increase retrograde transport of lysosomes in dendrites. Downregulation of MAP6 in TMEM106B knockdown neurons restores the balance of anterograde and retrograde lysosomal transport and thereby prevents loss of dendrites. To strengthen the link, we enhanced anterograde lysosomal transport by expressing dominant-negative Rab7-interacting lysosomal protein (RILP), which also rescues the dendrite loss in TMEM106B knockdown neurons. Thus, TMEM106B/MAP6 interaction is crucial for controlling dendritic trafficking of lysosomes, presumably by acting as a molecular brake for retrograde transport. Lysosomal misrouting may promote neurodegeneration in patients with *TMEM106B* risk variants.

3.2153 **Agonist-Induced GPCR Shedding from the Ciliary Surface Is Dependent on ESCRT-III and VPS4**
Soetedjo, L and Jin, H.
Current Biology, ***24(5)***, *509-518 (2014)*

Background
Membrane trafficking of G protein-coupled receptors (GPCRs) is crucial for temporal and spatial control of cell-surface GPCR signaling. Receptor internalization is a well-documented method cells use for regulating a wide variety of GPCRs following their exposure to agonists.
Results
We report that, upon agonist stimulation, a GPCR called vasoactive intestinal peptide receptor 2 (VPAC2) is shed, rather than being internalized, in vitro and in vivo, from the membrane of primary cilia—solitary hair-like organelles that project from the cell surface. VPAC2 is released into the extracellular milieu in the form of ciliary ectosomes that are devoid of exosome markers. The agonist-induced VPAC2 shedding is selective, as shown by the fact that other ciliary membrane proteins including two ciliary GPCRs are not shed with VPAC2. VPAC2 ectosome shedding is dependent on several components of endosomal sorting complexes required for transport (ESCRT), including a subset of ESCRT-III, VPS4, and LIP5. Agonist-stimulated VPAC2 is important for ciliary-ectosome generation because it allows VPS4 and LIP5 to transiently accumulate in primary cilia. Shedding of VPAC2 from the ciliary surface results in termination of intracellular VPAC2 signaling.
Conclusions
Agonist-induced GPCR shedding from the ciliary surface may represent an additional mode of GPCR trafficking and signal regulation.

3.2154 **Preferential secretion of inducible HSP70 by vitiligo melanocytes under stress**
Mosenson, J.A., Flood, K., Klarquist, J., Eby, J.M., Koshoffer, A., Boissy, R.E., Overbeck, A., Tung, R.C. and Le poole, L.C.
Pigment Cell & Melanoma Res., ***27(2)***, *209-220 (2014)*

Inducible HSP70 (HSP70i) chaperones peptides from stressed cells, protecting them from apoptosis. Upon extracellular release, HSP70i serves an adjuvant function, enhancing immune responses to bound peptides. We questioned whether HSP70i differentially protects control and vitiligo melanocytes from stress and subsequent immune responses. We compared expression of HSP70i in skin samples, evaluated the viability of primary vitiligo and control melanocytes exposed to bleaching phenols, and measured secreted HSP70i. We determined whether HSP70i traffics to melanosomes to contact immunogenic proteins by cell fractionation, western blotting, electron microscopy, and confocal microscopy. Viability of vitiligo and control melanocytes was equally affected under stress. However, vitiligo melanocytes secreted increased amounts of HSP70i in response to MBEH, corroborating with aberrant HSP70i expression in patient skin. Intracellular HSP70i colocalized with melanosomes, and more so in response to MBEH in vitiligo

melanocytes. Thus, whereas either agent is cytotoxic to melanocytes, MBEH preferentially induces immune responses to melanocytes.

3.2155 Soybean Proteomics
Hossain, Z. and Komatsu, S.
Methods in Mol. Biol., ***1072***, *315-331 (2014)*

Soybean, the world's most widely grown seed legume, is an important global source of vegetable oil and protein. Though, complete draft genome sequence of soybean is now available, but functional genomics studies remain in their infancy, as this agricultural legume species exhibits genetic constrains like genome duplications and self-incompatibilities. The techniques of proteomics provide much powerful tool for functional analysis of soybean. In the present review, an attempt has been made to summarize all significant contributions in the field of soybean proteomics. Special emphasis is given to subcellular proteomics in response to abiotic stresses for better understanding molecular basis of acquisition of stress tolerance mechanism. Detailed protocols of protein extraction, solubilization, fractionation of subcellular organelle, and proteins identification are explained for soybean proteomics. All this information would not only enrich us in understanding the plants response to environmental stressors but would also enable us to design genetically engineered stress tolerant soybean.

3.2156 Fluorescence-activated sorting of fixed nuclei: a general method for studying nuclei from specific cell populations that preserves post-translational modifications
Marion-Poll, L., Montalban, E., Munier, A., herve, D. and Giralult, J-A.
Eur. J. Neurosci., ***39***, *1234-1244 (2014)*

Long-lasting brain alterations that underlie learning and memory are triggered by synaptic activity. How activity can exert long-lasting effects on neurons is a major question in neuroscience. Signalling pathways from cytoplasm to nucleus and the resulting changes in transcription and epigenetic modifications are particularly relevant in this context. However, a major difficulty in their study comes from the cellular heterogeneity of brain tissue. A promising approach is to directly purify identified nuclei. Using mouse striatum we have developed a rapid and efficient method for isolating cell type-specific nuclei from fixed adult brain (fluorescence-activated sorting of fixed nuclei; FAST-FIN). Animals are quickly perfused with a formaldehyde fixative that stops enzymatic reactions and maintains the tissue in the state it was at the time of death, including nuclear localisation of soluble proteins such as GFP and differences in nuclear size between cell types. Tissue is subsequently dissociated with a Dounce homogeniser and nuclei prepared by centrifugation in an iodixanol density gradient. The purified fixed nuclei can then be immunostained with specific antibodies and analysed or sorted by flow cytometry. Simple criteria allow distinction of neurons and non-neuronal cells. Immunolabelling and transgenic mice that express fluorescent proteins can be used to identify specific cell populations, and the nuclei from these populations can be efficiently isolated, even rare cell types such as parvalbumin-expressing interneurons. FAST-FIN allows the preservation and study of dynamic and labile post-translational protein modifications. It should be applicable to other tissues and species, and allow study of DNA and its modifications.

3.2157 Membrane vesicles of Clostridium perfringens type A strains induce innate and adaptive immunity
Jiang, Y., Kong, Q., Roland, K.L. and Curtiss III, R.
Int. J. Med. Microbiol., ***304***, *431-443 (2014)*

Vesicle shedding from bacteria is a universal process in most Gram-negative bacteria and a few Gram-positive bacteria. In this report, we isolate extracellular membrane vesicles (MVs) from the supernatants of Gram-positive pathogen *Clostridium perfringens* (*C. perfringens*). We demonstrated vesicle production in a variety of virulent and nonvirulent type A strains. MVs did not contain alpha-toxin and NetB toxin demonstrated by negative reaction to specific antibody and absence of specific proteins identified by LC-MS/MS. *C. perfringens* MVs contained DNA components such as 16S ribosomal RNA gene (16S rRNA), alpha-toxin gene (*plc*) and the perfringolysin O gene (*pfoA*) demonstrated by PCR. We also identified a total of 431 proteins in vesicles by 1-D gel separation and LC-MS/MS analysis. In vitro studies demonstrated that vesicles could be internalized into murine macrophage RAW264.7 cells without direct cytotoxicity effects, causing release of inflammation cytokines including granulocyte colony stimulating factor (G-CSF), tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), which could also be detected in mice injected with MVs through intraperitoneal (i.p.) route. Mice immunized with *C. perfringens* MVs produced high titer IgG, especially IgG1, antibodies against *C. perfringens* membrane

proteins. However, this kind of antibody could not provide protection in mice following challenge, though it could slightly postpone the time of death. Our results indicate that release of MVs from *C. perfringens* could provide a previously unknown mechanism to induce release of inflammatory cytokines, especially TNF-α, these findings may contribute to a better understanding of the pathogenesis of *C. perfringens* infection.

3.2158 Characterization of protective extracellular membrane-derived vesicles produced by Streptococcus pneumonia

Olaya-Abril, A., Prados-Rosales, R., McConnell, M.J., martin-Pena, R., Gonzalez-Reyes, J.A., Jimenez-Munguia, I., Gomez-Gascon, L., Fernandez, J., Luque-garcia, J.L., Garcia-Lidon, C., Estevez, H., pachon, J., Obando, I., Casadevall, A., Profski. L-A. and Rodriguez-Ortega, M.J.
*J. Proteomics, **106**, 46-60 (2014)*

Extracellular vesicles are produced by many pathogenic microorganisms and have varied functions that include secretion and release of microbial factors, which contribute to virulence. Very little is known about vesicle production by Gram-positive bacteria, as well as their biogenesis and release mechanisms. In this work, we demonstrate the active production of vesicles by *Streptococcus pneumoniae* from the plasma membrane, rather than being a product from cell lysis. We biochemically characterized them by proteomics and fatty acid analysis, showing that these vesicles and the plasma membrane resemble in essential aspects, but have some differences: vesicles are more enriched in lipoproteins and short-chain fatty acids. We also demonstrate that these vesicles act as carriers of surface proteins and virulence factors. They are also highly immunoreactive against human sera and induce immune responses that protect against infection. Overall, this work provides insights into the biology of this important Gram-positive human pathogen and the role of extracellular vesicles in clinical applications.

3.2159 Angiogenin interacts with the plasminogen activation system at the cell surface of breast cancer cells to regulate plasmin formation and cell migration

Dutta, S., Bandyopadhyay, C., Bottero, V., Veettil, M.V., Wilson, L., Pins, M.R., Johnson, K.E., Warshall, C. and Chandran, B.
*Mol. Oncol., **8**, 483-507 (2014)*

Angiogenin (ANG), a 14-kDa pro-angiogenic secreted protein, has been shown to play a role in cell migration and tumor invasion, which involve proteolytic cleavage of plasminogen to generate plasmin. However, the mechanism by which ANG regulates plasmin formation and cell migration was not known. Our studies here detected elevated levels of secreted and cell surface-bound ANG in highly invasive metastatic breast cancer cells. ANG was also detected at very high levels in the tumor cells in infiltrating ductal carcinomas. By immunofluorescence and immunoprecipitation analysis, ANG was detected at the leading edges of the cell surfaces where it colocalized and interacted with members of the plasminogen activation system (PAS) such as annexin A2 (A2), calpactin (S100-A10) and urokinase plasminogen activator receptor (uPAR). Analysis of lipid raft (LR) and non-lipid raft (NLR) regions of the cell membranes showed the predominance of ANG, A2 and S100-A10 in the LR regions. In contrast, uPAR was detected predominantly in the NLR fractions, suggesting that ANG interacts with uPAR at the junctions of LR and NLR regions. ANG knockdown in T47D and MDA-MB-231 breast cancer cell lines did not affect the cellular expression of A2, S100-A10 and uPAR but decreased cell migration and plasmin formation. Neutralization of ANG with monoclonal antibodies similarly decreased the migration of MDA-MB-231 cells. In the presence of ANG, uPAR was observed to interact with uPA, which is necessary for plasmin formation. Conversely, in the absence of ANG, uPAR did not interact with uPA and FAK and Src kinases were observed to be dephosphorylated. Exogenous addition of recombinant ANG to ANG knocked down MDA-MB-231 cells restored FAK phosphorylation, uPAR interactions with uPA, plasmin formation as well as migration of these cells. Taken together, our results identified a novel role for ANG as a member of the uPAR interactome that facilitates the interaction of uPAR with uPA, leading to plasmin formation and cell migration necessary for tumor invasion and metastasis of breast cancer cells.

3.2160 Positional Assembly of Enzymes on Bacterial Outer Membrane Vesicles for Cascade Reactions

Park, M., Sun, Q., Liu, F., DeLisa, M.P. and Chen, W.
*PloS One, **9(5)**, e97103 (2014)*

The systematic organization of enzymes is a key feature for the efficient operation of cascade reactions in nature. Here, we demonstrate a facile method to create nanoscale enzyme cascades by using engineered bacterial outer membrane vesicles (OMVs) that are spheroid nanoparticles (roughly 50 nm in diameter)

produced by Gram-negative bacteria during all phases of growth. By taking advantage of the fact that OMVs naturally contain proteins found in the outer cell membrane, we displayed a trivalent protein scaffold containing three divergent cohesin domains for the position-specific presentation of a three-enzyme cascade on OMVs through a truncated ice nucleation protein anchoring motif (INP). The positional assembly of three enzymes for cellulose hydrolysis was demonstrated. The enzyme-decorated OMVs provided synergistic cellulose hydrolysis resulting in 23-fold enhancement in glucose production than free enzymes.

3.2161 Heterologous Src Homology 4 Domains Support Membrane Anchoring and Biological Activity of HIV-1 Nef

Geist, M.M., Pan, X., Bender, S., Bartenschlager, R., Nickel, W. and Fackler, O.T.
J. Biol. Chem., ***289(20)***, *14030-14044 (2014)*

The HIV-1 pathogenicity factor Nef enhances viral replication by modulation of multiple host cell transport and signaling pathways. Nef associates with membranes via an N-terminal Src homology 4 (SH4) domain, and membrane association is believed to be essential for its biological functions. At which subcellular site(s) Nef exerts its different functions and how kinetics of membrane interactions contribute to its biological activity are unknown. To address how specific characteristics of Nef membrane association affect its biological properties, the SH4 domain of Nef was replaced by heterologous membrane targeting domains. The use of a panel of heterologous SH4 domains resulted in chimeric Nef proteins with distinct steady state subcellular localization, membrane association efficiency, and anterograde transport routes. Irrespective of these modifications, cardinal Nef functions affecting host cell vesicular transport and actin dynamics were fully preserved. In contrast, stable targeting of Nef to the surface of mitochondria, peroxisomes, or the Golgi apparatus, and thus prevention of plasma membrane delivery, caused potent and broad loss of Nef activity. These results support the concept that Nef adopts its active conformation in the membrane-associated state but exclude that membrane-associated Nef simply acts by recruiting soluble factors independently of its local microenvironment. Rather than its steady state subcellular localization or membrane affinity, the ability to undergo dynamic anterograde and internalization cycles appear to determine Nef function. These results reveal that functional membrane interactions of Nef underlie critical spatiotemporal regulation and suggest that delivery to distinct subcellular sites via such transport cycles provides the basis for the multifunctionality of Nef.

3.2162 Plac8 Links Oncogenic Mutations to Regulation of Autophagy and Is Critical to Pancreatic Cancer Progression

Kinsey, C., Balakrishnan, V., O'Dell, M.R., Huang, J.L., Newman, L., Whitney-Miller, C.L., hezel, A.F. and Land, H.
Cell Reports, ***7***, *1143-1155 (2014)*

Mutations in *p53* and *RAS* potently cooperate in oncogenic transformation, and correspondingly, these genetic alterations frequently coexist in pancreatic ductal adenocarcinoma (PDA) and other human cancers. Previously, we identified a set of genes synergistically activated by combined *RAS* and *p53* mutations as frequent downstream mediators of tumorigenesis. Here, we show that the synergistically activated gene *Plac8* is critical for pancreatic cancer growth. Silencing of *Plac8* in cell lines suppresses tumor formation by blocking autophagy, a process essential for maintaining metabolic homeostasis in PDA, and genetic inactivation in an engineered mouse model inhibits PDA progression. We show that Plac8 is a critical regulator of the autophagic machinery, localizing to the lysosomal compartment and facilitating lysosome-autophagosome fusion. *Plac8* thus provides a mechanistic link between primary oncogenic mutations and the induction of autophagy, a central mechanism of metabolic reprogramming, during PDA progression.

3.2163 Adiponectin is partially associated with exosomes in mouse serum

Phoonsawat, W., Aoki-Yoshida, A., Tsuruta, T. and Sonoyama, K.
Biochem. Biophys. Res. Comm., ***448***, *261-266 (2014)*

Exosomes are membrane vesicles 30–120 nm in diameter that are released by many cell types and carry a cargo of proteins, lipids, mRNA, and microRNA. Cultured adipocytes reportedly release exosomes that may play a role in cell-to-cell communication during the development of metabolic diseases. However, the characteristics and function of exosomes released from adipocytes *in vivo* remain to be elucidated. Clearly, adipocyte-derived exosomes could exist in the circulation and may be associated with adipocyte-specific proteins such as adipocytokines. We isolated exosomes from serum of mice by differential centrifugation

and analyzed adiponectin, leptin, and resistin in the exosome fraction. Western blotting detected adiponectin but no leptin and only trace amounts of resistin in the exosome fraction. The adiponectin signal in the exosome fraction was decreased by proteinase K treatment and completely quenched by a combination of proteinase K and Triton X-100. Quantitative ELISA showed that the exosome fraction contains considerable amounts of adiponectin, but not leptin or resistin. The concentration of adiponectin in the serum and the ratio of adiponectin to total protein in the exosome fraction were lower in obese mice than in lean mice. These results suggest that a portion of adiponectin exists as a transmembrane protein in the exosomes in mouse serum. We propose adiponectin as a marker of exosomes released from adipocytes *in vivo*.

3.2164 Peroxisomal membrane channel Pxmp2 in the mammary fat pad is essential for stromal lipid homeostasis and for development of mammary gland epithelium in mice
Vapola, M.H., Rokka, A., Sormunen, R.T., Alhonen, L., Schmitz, W., Conzelmann, E., Wärri, A., Grunau, S., Antonenkov, V.D. and Hiltunen, J.K.
*Developmental Biol., **391**, 66-80 (2014)*

To understand the functional role of the peroxisomal membrane channel Pxmp2, mice with a targeted disruption of the *Pxmp2* gene were generated. These mice were viable, grew and bred normally. However, *Pxmp2*−/− female mice were unable to nurse their pups. Lactating mammary gland epithelium displayed secretory lipid droplets and milk proteins, but the size of the ductal system was greatly reduced. Examination of mammary gland development revealed that retarded mammary ductal outgrowth was due to reduced proliferation of epithelial cells during puberty. Transplantation experiments established the *Pxmp2*−/− mammary stroma as a tissue responsible for suppression of epithelial growth. Morphological and biochemical examination confirmed the presence of peroxisomes in the mammary fat pad adipocytes, and functional Pxmp2 was detected in the stroma of wild-type mammary glands. Deletion of Pxmp2 led to an elevation in the expression of peroxisomal proteins in the mammary fat pad but not in liver or kidney of transgenic mice. Lipidomics of *Pxmp2*−/− mammary fat pad showed a decrease in the content of myristic acid (C_{14}), a principal substrate for protein myristoylation and a potential peroxisomal β-oxidation product. Analysis of complex lipids revealed a reduced concentration of a variety of diacylglycerols and phospholipids containing mostly polyunsaturated fatty acids that may be caused by activation of lipid peroxidation. However, an antioxidant-containing diet did not stimulate mammary epithelial proliferation in *Pxmp2*−/− mice.
The results point to disturbances of lipid metabolism in the mammary fat pad that in turn may result in abnormal epithelial growth. The work reveals impaired mammary gland development as a new category of peroxisomal disorders.

3.2165 Conformational targeting of intracellular Aβ oligomers demonstrates their pathological oligomerization inside the endoplasmic reticulum
Meli, G., lecci, A., Manca, A., Krako, N., Albertini, V., Benussi, L., Ghidoni, R. and Cattaneo, A.
*Nature Comm., **5**:3867 (2014)*

Aβ oligomers (AβOs) are crucially involved in Alzheimer's Disease (AD). However, the lack of selective approaches for targeting these polymorphic Aβ assemblies represents a major hurdle in understanding their biosynthesis, traffic and actions in living cells. Here, we established a subcellularly localized conformational-selective interference (CSI) approach, based on the expression of a recombinant antibody fragment against AβOs in the endoplasmic reticulum (ER). By CSI, we can control extra- and intracellular pools of AβOs produced in an AD-relevant cell model, without interfering with the maturation and processing of the Aβ precursor protein. The anti-AβOs intrabody selectively intercepts critical AβO conformers in the ER, modulating their assembly and controlling their actions in pathways of cellular homeostasis and synaptic signalling. Our results demonstrate that intracellular Aβ undergoes pathological oligomerization through critical conformations formed inside the ER. This establishes intracellular AβOs as key targets for AD treatment and presents CSI as a potential targeting strategy.

3.2166 hnRNP L and NF90 Interact with Hepatitis C Virus 5'-Terminal Untranslated RNA and Promote Efficient Replication
Li, Y., Masaki, T., Shimakami, T and Lemon, S.M.
*J. Virol., **88(13)**, 7199-7209 (2014)*

The 5′-terminal sequence of the hepatitis C virus (HCV) positive-strand RNA genome is essential for viral replication. Critical host factors, including a miR-122/Ago2 complex and poly(rC)-binding protein 2

(PCBP2), associate with this RNA segment. We used a biotinylated RNA pulldown approach to isolate host factors binding to the HCV 5′ terminal 47 nucleotides and, in addition to Ago2 and PCBP2, identified several novel proteins, including IGF2BP1, hnRNP L, DHX9, ADAR1, and NF90 (ILF3). PCBP2, IGF2BP1, and hnRNP L bound single-stranded RNA, while DHX9, ADAR1, and NF90 bound a cognate double-stranded RNA bait. PCBP2, IGF2BP1, and hnRNP L binding were blocked by preannealing the single-stranded RNA bait with miR-122, indicating that they bind the RNA in competition with miR-122. However, IGF2BP1 binding was also inhibited by high concentrations of heparin, suggesting that it bound the bait nonspecifically. Among these proteins, small interfering RNA-mediated depletion of hnRNP L and NF90 significantly impaired viral replication and reduced infectious virus yields without substantially affecting HCV internal ribosome entry site-mediated translation. hnRNP L and NF90 were found to associate with HCV RNA in infected cells and to coimmunoprecipitate with NS5A in an RNA-dependent manner. Both also associate with detergent-resistant membranes where viral replication complexes reside. We conclude that hnRNP and NF90 are important host factors for HCV replication, at least in cultured cells, and may be present in the replication complex.

3.2167 Involvement of Hepatitis C Virus NS5A Hyperphosphorylation Mediated by Casein Kinase I-α in Infectious Virus Production

Masaki, T., Matsunaga, S., Takahashi, H., Nakashima, K., Kimura, Y., Ito, M., Matsuda, M., Murayama, A., Kato, T., Hirano, H., Endo, Y., Lemon, S.M., Wakita, t., Sawasaki, T. and Suzuki, T.
*J. Virol.,**88(13)**, 7541-7555 (2014)*

Nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) possesses multiple functions in the viral life cycle. NS5A is a phosphoprotein that exists in hyperphosphorylated and basally phosphorylated forms. Although the phosphorylation status of NS5A is considered to have a significant impact on its function, the mechanistic details regulating NS5A phosphorylation, as well as its exact roles in the HCV life cycle, are still poorly understood. In this study, we screened 404 human protein kinases via in vitro binding and phosphorylation assays, followed by RNA interference-mediated gene silencing in an HCV cell culture system. Casein kinase I-α (CKI-α) was identified as an NS5A-associated kinase involved in NS5A hyperphosphorylation and infectious virus production. Subcellular fractionation and immunofluorescence confocal microscopy analyses showed that CKI-α-mediated hyperphosphorylation of NS5A contributes to the recruitment of NS5A to low-density membrane structures around lipid droplets (LDs) and facilitates its interaction with core protein and the viral assembly. Phospho-proteomic analysis of NS5A with or without CKI-α depletion identified peptide fragments that corresponded to the region located within the low-complexity sequence I, which is important for CKI-α-mediated NS5A hyperphosphorylation. This region contains eight serine residues that are highly conserved among HCV isolates, and subsequent mutagenesis analysis demonstrated that serine residues at amino acids 225 and 232 in NS5A (genotype 2a) may be involved in NS5A hyperphosphorylation and hyperphosphorylation-dependent regulation of virion production. These findings provide insight concerning the functional role of NS5A phosphorylation as a regulatory switch that modulates its multiple functions in the HCV life cycle.

3.2168 The fronds tonoplast quantitative proteomic analysis in arsenic hyperaccumulator Pteris vittata L.

Shen, H., He, Z., Yan, H., Xing, Z., Chen, Y., Xu, W., Xu, W. and Ma, M.
*J. Proteomics, **105**, 46-57 (2014)*

Pteris vittata, the first known arsenic hyperaccumulating plant, can accumulate very high concentration arsenic in its aboveground tissues, while low in roots. Previous studies have suggested that arsenic vacuole compartmentalization may play an important role in the arsenic-hyperaccumulation in *P. vittata*, but the mechanism(s) of arsenic transport to vacuole are largely unknown. We obtained tonoplast isolated from fronds of *P. vittata* sporophyte grown under minus and 1 mM arsenate for 3 weeks by iodixanol step gradient centrifugation method, and then used TMPP protein labeling technology followed by liquid chromatography–a linear ion trap-Orbitrap hybrid mass spectrometer analysis for the quantitative detection of proteins. And we designed and used an "artificial" database for database searching. In total, 56 tonoplast proteins were identified; more than 70% of them were transport proteins. Under arsenate treatment, one TDT transporter protein, a member of the TerC family and a PDR-like protein were upregulated differentially. While V-ATPase subunits c, E, and G, and V-PPase, were downregulated. Additionally, the identified tonoplast proteins in our present study provide an informative basis for arsenic carriers or channels and help to clarify the regulation of tonoplast arsenic transport processes in *P. vittata*.

3.2169 Intracellular gene delivery is dependent on the type of non-viral carrier and defined by the cell surface glycosaminoglycans

Normani, A., Hyvönen, Z., Pulkkinen, E., Hiekkala, M. and Ruponen, M.
*J. Controlled Release, **187**, 59-65 (2014)*

Intracellular limiting steps and molecules involved in internalization and intracellular routing of non-viral gene delivery systems are still poorly understood. In this study, the intracellular kinetics of three different gene delivery systems calcium phosphate precipitates (CaP), polyethyleneimine (PEI) and N-[1-(2,3-dioleyl)propyl]-N,N,N-trimethylammonium chloride (DOTAP)) were quantified at cellular, nuclear, transcriptional and translational levels by using qRT-PCR. Additionally, a role of cell surface glycosaminoglycans (GAGs) was evaluated by performing the aforementioned studies in cells devoid of GAGs (pgsB-618) and cells lacking heparan sulphate (HS). The obtained data showed that the intracellular kinetics was dependent on the type of gene carrier and the weakest intracellular step varied between the carriers; rapid elimination of cell-associated pDNA in CaP, nuclear uptake in DOTAP and transcriptional and translational events in PEI mediated transfections. Overall, neither the amount of cell- nor nuclear associated pDNA correlated with transgene expression but the mRNA expression of the transgene correlated well with the expression at protein level. The nuclear uptake of pDNA in all cases was rapid and efficient thus indicating that the post-nuclear processes including transcription and translation steps have a critical role in defining the efficiency of non-viral gene delivery systems. Our study demonstrated that cell-surface GAGs are not essential for cell surface binding and internalization of gene delivery complexes, but they are able to define the intracellular routing of the complexes by leading them to pathways with high pDNA elimination.

3.2170 A protocol for the subcellular fractionation of Saccharomyces cerevisiae using nitrogen cavitation and density gradient centrifugation

Wang, Y., Lilley, K.S. and Oliver, S.G.
*Yeast, **31**, 127-135 (2014)*

Most protocols for yeast subcellular fractionation involve the use of mechanical shear forces to lyse the spheroplasts produced by the enzymatic digestion of the *Saccharomyces cerevisiae* cell wall. These mechanical homogenization procedures often involve the manual use of devices such as the Dounce homogenizer, and so are very operator-dependent and, in consequence, lack reproducibility. Here, we report a highly reproducible method of homogenizing yeast cells based on nitrogen cavitation. This has been optimized to allow efficient release of subcellular compartments that show a high degree of integrity. The protocol remains effective and reproducible across a range of sample volumes and buffer environments. The subsequent separation method, which employs both sucrose and iodixanol density gradients, has been developed to resolve the major membrane-bound compartments of *S. cerevisiae*. We present an integrated protocol that is fast, facile, robust and efficient and that will enable 'omics' studies of the subcellular compartments of *S. cerevisiae* and other yeasts.

3.2171 HIV-1 protease-induced apoptosis

Rumlova, M., Krizova, I., Keprova, A., Hadravova, R., Dolezal, M., Strohalmova, K., Pichova, I., Hajek, M. and Ruml, T.
*Retrovirology, **11**:37 (2014)*

Background
Apoptosis is one of the presumptive causes of $CD4^+$ T cell depletion during HIV infection and progression to AIDS. However, the precise role of HIV-1 in this process remains unexplained. HIV-1 protease (PR) has been suggested as a possible factor, but a direct link between HIV-1 PR enzymatic activity and apoptosis has not been established.
Results
Here, we show that expression of active HIV-1 PR induces death in HeLa and HEK-293 cells *via* the mitochondrial apoptotic pathway. This conclusion is based on *in vivo* observations of the direct localization of HIV-1 PR in mitochondria, a key player in triggering apoptosis. Moreover, we observed an HIV-1 PR concentration-dependent decrease in mitochondrial membrane potential and the role of HIV-1 PR in activation of caspase 9, PARP cleavage and DNA fragmentation. In addition, *in vitro* data demonstrated that HIV-1 PR mediates cleavage of mitochondrial proteins Tom22, VDAC and ANT, leading to release of AIF and Hsp60 proteins. By using yeast two-hybrid screening, we also identified a new HIV-1 PR interaction partner, breast carcinoma-associated protein 3 (BCA3). We found that BCA3

accelerates p53 transcriptional activity on the *bax* promoter, thus elevating the cellular level of pro-apoptotic Bax protein.

Conclusion

In summary, our results describe the involvement of HIV-1 PR in apoptosis, which is caused either by a direct effect of HIV-1 PR on mitochondrial membrane integrity or by its interaction with cellular protein BCA3.

3.2172 Potential candidate camelid antibodies for the treatment of protein-misfolding diseases

David, M.A., Jones, D.R. and Tayebi, M.
J. Neuroimmunol., ***272***, *76-85 (2014)*

Protein-misfolding diseases (PMDs), including Alzheimer's disease would potentially reach epidemic proportion if effective ways to diagnose and treat them were not developed. The quest for effective therapy for PMDs has been ongoing for decades and some of the technologies developed so far show great promise. We report here the development of antibodies by immunization of camelids with prion (PrioV3) and Alzheimer's (PrioAD12, 13 & 120) disease-derived brain material. We show that anti-PrP antibody transmigration across the blood‑brain barrier (BBB) was inhibited with phosphatidylinositol-specific phospholipase C (PIPLC). Our camelid anti-prion antibody was also shown to permanently abrogate prion replication in a prion-permissive cell line after crossing the artificial BBB. Furthermore, anti-A β /tau antibodies were able to bind their specific immunogens with ELISA and immunohistochemistry. Finally, both PrioV3 and PrioAD12 were shown to co-localize with Lamp-1, a marker of late endosomal/lysosomal compartments.

These antibodies could prove to be a valuable tool for the neutralization/clearance of PrP^{Sc}, A β and tau proteins in cellular compartments of affected neurons and could potentially have wider applicability for the treatment of PMDs.

3.2173 Specific recycling receptors are targeted to the immune synapse by the intraflagellar transport system

Finetti, F., Patrussi, L., Masi, G., Onnis, A., Galgano, D., Lucherini, O.M., Pazour, G. and Baldari, C.T.
J. Cell Sci., ***127***, *1924-1937 (2014)*

T cell activation requires sustained signaling at the immune synapse, a specialized interface with the antigen-presenting cell (APC) that assembles following T cell antigen receptor (TCR) engagement by major histocompatibility complex (MHC)-bound peptide. Central to sustained signaling is the continuous recruitment of TCRs to the immune synapse. These TCRs are partly mobilized from an endosomal pool by polarized recycling. We have identified IFT20, a component of the intraflagellar transport (IFT) system that controls ciliogenesis, as a central regulator of TCR recycling to the immune synapse. Here, we have investigated the interplay of IFT20 with the Rab GTPase network that controls recycling. We found that IFT20 forms a complex with Rab5 and the TCR on early endosomes. IFT20 knockdown (IFT20KD) resulted in a block in the recycling pathway, leading to a build-up of recycling TCRs in $Rab5^{+}$ endosomes. Recycling of the transferrin receptor (TfR), but not of CXCR4, was disrupted by IFT20 deficiency. The IFT components IFT52 and IFT57 were found to act together with IFT20 to regulate TCR and TfR recycling. The results provide novel insights into the mechanisms that control TCR recycling and immune synapse assembly, and underscore the trafficking-related function of the IFT system beyond ciliogenesis.

3.2174 Parkinson's disease-linked human PARK9/ATP13A2 maintains zinc homeostasis and promotes α-Synuclein externalization via Exosomes

Kong, S.M.Y., Chan, B.K.K., Park, J-S., Hill, K.J., Aitken, J.B., Cottle, L., Farghaian, H., Cole, A.R., Lay, P.A., Sue, C.M. and Cooper, A.A.
Hum. Mol. Genet., ***23(11)***, *2816-2833 (2014)*

α-Synuclein plays a central causative role in Parkinson's disease (PD). Increased expression of the P-type ATPase ion pump PARK9/ATP13A2 suppresses α-Synuclein toxicity in primary neurons. Our data indicate that ATP13A2 encodes a zinc pump; neurospheres from a compound heterozygous $ATP13A2^{-/-}$ patient and ATP13A2 knockdown cells are sensitive to zinc, whereas ATP13A2 over-expression in primary neurons confers zinc resistance. Reduced ATP13A2 expression significantly decreased vesicular zinc levels, indicating ATP13A2 facilitates transport of zinc into membrane-bound compartments or vesicles. Endogenous ATP13A2 localized to multi-vesicular bodies (MVBs), a late endosomal compartment located at the convergence point of the endosomal and autophagic pathways. Dysfunction in MVBs can cause a range of detrimental effects including lysosomal dysfunction and impaired delivery of

endocytosed proteins/autophagy cargo to the lysosome, both of which have been observed in cells with reduced ATP13A2 function. MVBs also serve as the source of intra-luminal nanovesicles released extracellularly as exosomes that can contain a range of cargoes including α-Synuclein. Elevated ATP13A2 expression reduced intracellular α-Synuclein levels and increased α-Synuclein externalization in exosomes >3-fold whereas ATP13A2 knockdown decreased α-Synuclein externalization. An increased export of exosome-associated α-Synuclein may explain why surviving neurons of the substantia nigra pars compacta in sporadic PD patients were observed to over-express ATP13A2. We propose ATP13A2's modulation of zinc levels in MVBs can regulate the biogenesis of exosomes capable of containing α-Synuclein. Our data indicate that ATP13A2 is the first PD-associated gene involved in exosome biogenesis and indicates a potential neuroprotective role of exosomes in PD.

3.2175 Interaction of Integrin β4 With S1P Receptors in S1P- and HGF-Induced Endothelial Barrier Enhancement

Ni, X., Epshtein, Y., Chen, W., Zhou, ts., Xie, L., Garcia, J.G.N. and jacobson, J.R.
J. Cell. Biochem., ***115(6)***, *1187-1195 (2014)*

We previously reported sphingosine 1-phosphate (S1P) and hepatocyte growth factor (HGF) augment endothelial cell (EC) barrier function and attenuate murine acute lung inury (ALI). While the mechanisms underlying these effects are not fully understood, S1P and HGF both transactivate the S1P receptor, S1PR1 and integrin β4 (ITGB4) at membrane caveolin-enriched microdomains (CEMs). In the current study, we investigated the roles of S1PR2 and S1PR3 in S1P/HGF-mediated EC signaling and their associations with ITGB4. Our studies confirmed ITGB4 and S1PR2/3 are recruited to CEMs in human lung EC in response to either S1P (1 μM, 5 min) or HGF (25 ng/ml, 5 min). Co-immunoprecipitation experiments identified an S1P/HGF-mediated interaction of ITGB4 with both S1PR2 and S1PR3. We then employed an in situ proximity ligation assay (PLA) to confirm a direct ITGB4–S1PR3 association induced by S1P/HGF although a direct association was not detectable between S1PR2 and ITGB4. S1PR1 knockdown (siRNA), however, abrogated S1P/HGF-induced ITGB4–S1PR2 associations while there was no effect on ITGB4–S1PR3 associations. Moreover, PLA confirmed a direct association between S1PR1 and S1PR2 induced by S1P and HGF. Finally, silencing of S1PR2 significantly attenuated S1P/HGF-induced EC barrier enhancement as measured by transendothelial resistance while silencing of S1PR3 significantly augmented S1P/HGF-induced barrier enhancement. These results confirm an important role for S1PR2 and S1PR3 in S1P/HGF-mediated EC barrier responses that are associated with their complex formation with ITGB4. Our findings elucidate novel mechanisms of EC barrier regulation that may ultimately lead to new therapeutic targets for disorders characterized by increased vascular permeability including ALI.

3.2176 Regulation of TrkB receptor translocation to lipid rafts by adenosine A2A receptors and its functional implications for BDNF-induced regulation of synaptic plasticity

Assaife-Lopes, N., Sousa, V.C., pereira, D.B., Ribeiro, J.A. and Sebastiao, A.M.
Purinergic Signalling, ***10(2)***, *251-267 (2014)*

Brain-derived neurotrophic factor (BDNF) signalling is critical for neuronal development and transmission. Recruitment of TrkB receptors to lipid rafts has been shown to be necessary for the activation of specific signalling pathways and modulation of neurotransmitter release by BDNF. Since TrkB receptors are known to be modulated by adenosine A_{2A} receptor activation, we hypothesized that activation of A_{2A} receptors could influence TrkB receptor localization among different membrane microdomains. We found that adenosine A_{2A} receptor agonists increased the levels of TrkB receptors in the lipid raft fraction of cortical membranes and potentiated BDNF-induced augmentation of phosphorylated TrkB levels in lipid rafts. Blockade of the clathrin-mediated endocytosis with monodansyl cadaverine (100 μM) did not modify the effects of the A_{2A} receptor agonists, but significantly impaired BDNF effects on TrkB recruitment to lipid rafts. The effect of A_{2A} receptor activation in TrkB localization was mimicked by 5 μM forskolin, an adenylyl cyclase activator. Also, it was blocked by the PKA inhibitors Rp-cAMPs and PKI-(14-22) and by the Src-family kinase inhibitor PP2. Moreover, removal of endogenous adenosine or disruption of lipid rafts reduced BDNF stimulatory effects on glutamate release from cortical synaptosomes. Lipid raft integrity was also required for the effects of BDNF upon hippocampal long-term potentiation at CA1 synapses. Our data demonstrate, for the first time, a BDNF-independent recruitment of TrkB receptors to lipid rafts, induced by the activation of adenosine A_{2A} receptors, with functional consequences for TrkB phosphorylation and BDNF-induced modulation of neurotransmitter release and hippocampal plasticity.

3.2177 Exosomes and their role in CNS viral infections

Sampey, G.C., Meyering, S.S., Zadeh, M.A., Saifuddin, M., Hakami, R. and Kashanchi, F.

J. Neurovirol., ***20(3)***, *199-208 (2014)*

Exosomes are small membrane-bound vesicles that carry biological macromolecules from the site of production to target sites either in the microenvironment or at distant sites away from the origin. Exosomal content of cells varies with the cell type that produces them as well as environmental factors that alter the normal state of the cell such as viral infection. Human DNA and RNA viruses alter the composition of host proteins as well as incorporate their own viral proteins and other cargo into the secreted exosomes. While numerous viruses can infect various cell types of the CNS and elicit damaging neuropathologies, few have been studied for their exosomal composition, content, and function on recipient cells. Therefore, there is a pressing need to understand how DNA and RNA viral infections in CNS control exosomal release. Some of the more recent studies including HIV-1, HTLV-1, and EBV-infected B cells indicate that exosomes from these infections contain viral miRNAs, viral transactivators, and a host of cytokines that can control the course of infection. Finally, because exosomes can serve as vehicles for the cellular delivery of proteins and RNA and given that the blood-brain barrier is a formidable challenge in delivering therapeutics to the brain, exosomes may be able to serve as ideal vehicles to deliver protein or RNA-based therapeutics to the brain.

3.2178 **Sulfatide-mediated control of extracellular matrix-dependent oligodendrocyte maturation**
Baron, W., Biljard, M., Nomden, A., de Jonge, J.C., Teunissen, C. and Hoekstra, D.
Glia, ***62(6)****, 927-942 (2014)*

In the central nervous system, the extracellular matrix (ECM) compound laminin-2, present on developing axons, is essential in regulating oligodendrocyte (OLG) maturation. For example, laminin-2 is involved in mediating interactions between integrins and growth factors, initially localizing in separate membrane microdomains. The galactosphingolipid sulfatide is an important constituent of these microdomains and may serve as a receptor for laminin-2. Here, we investigated whether sulfatide interferes with ECM–integrin interactions and, in this manner, modulates OLG maturation. Our data reveal that disruption of laminin-2–sulfatide interactions impeded OLG differentiation and myelin-like membrane formation. On laminin-2, but not on (re)myelination-inhibiting fibronectin, sulfatide laterally associated with integrin α6 in membrane microdomains. Sulfatide was partly excluded from membrane microdomains on fibronectin, thereby likely precluding laminin-2-mediated myelination. Anti-sulfatide antibodies disrupted integrin α6-PDGFαR interactions on laminin-2 and induced demyelination in myelinated spheroid cultures, but intriguingly stimulated myelin-like membrane formation on fibronectin. Taken together, these findings highlight the importance of laminin–sulfatide interactions in the formation of functional membrane microdomains essential for myelination. Thus, laminin–sulfatide interactions might control the asynchronous localized differentiation of OLGs, thereby allowing myelination to be triggered by axonal demand. Given the accumulation of fibronectin in multiple sclerosis lesions, the findings also provide a molecular rationale for the potential of anti-sulfatide antibodies to trigger quiescent endogenous OLG progenitor cells in axon remyelination.

3.2179 **Mo1751 CGMP-Dependent Kinase 2, Na+/H+ Regulatory Factor 2, and Na+/H+ Exchanger Isoform 3 Dynamically Assemble Within Lipid Rafts in Murine Small Intestinal Brush Border Membrane**
Luo, M., Liu, Y., Riederer, B., Patrucco, E., Hofman, F., Donowitz, M., Tian, D., Yun, C., de Jonge, H., Lamprecht, G. and Seidler, U.
Gastroenterology, ***146(5)****, Suppl. 1, S651-S652 (2014)*

Background: Trafficking, brush border membrane (BBM) retention, and signal-specific regulation of the Na+/H+ exchanger 3 (NHE3) is regulated by the Na+/H+ Exchanger Regulatory Factor (NHERF) family of PDZ-adapter proteins, which enables the formation of multiprotein complexes. We previously reported a differential association of the NHERFs to the lipid raft and non-raft fraction of NHE3 in murine intestinal BBM, with NHERF2 being strongly lipid raft associated. Aim: This study was undertaken to explore the association of NHE3 and NHERF2 with the cGMP-dependent kinase II (cGKII), a key enzyme in hormonal and toxinmediated inhibition of NHE3, within lipid rafts, and to assess the effect of stimulating this signal transduction pathway on the raft assembly of NHE3, NHERF2 and cGKII. Methods: Murine BBM was isolated from wild type, NHE3-deficient, cGMP-kinase II-deficient, and NHERF2-deficient mice before and after intestinal application of a heat-stable Escherichia coli toxin (STA) analogue in vivo. Lipid raft and non-raft fractions were separated by Optiprep density gradient centrifugation of Triton X solubilised isolated small intestinal BBM. The raft-associated and non-raft proteins were precipitated and studied by Western analysis.

Results: NHE3 and NHERF2 were strongly lipid raft associated, and cGMP-dependent kinase II, which together with NHERF2 is essential for guanylin/STa-mediated NHE3 inhibition, was almost exclusively lipid-raft associated. While NHE3 and NHERF2 were always observed in the same lipid raft fraction(s), a part of the cGKII was found in lipid rafts of lower specific weight. The application of an oral STa-analogue to the mice and subsequent small intestinal BBM isolation and lipid raft flotation assay demonstrated a redistribution of cGKII, NHE3 and NHERF2 in a dynamic fashion. Conclusion: The differential association of the NHERFs, as well as kinases, with the raft-associated and the non-raft fraction of NHE3 in the brush border membrane is likely one component of the differential and signal-specific NHE3 regulation by the different NHERFs. Many players of the signalling pathway for the guanylin analoges via cGMP-dependent kinase II, leading to NHE3 inhibition, are associated with lipid rafts in the murine small intestine.

3.2180 Oxysterol-Binding Protein Is a Phosphatidylinositol 4-Kinase Effector Required for HCV Replication Membrane Integrity and Cholesterol Trafficking

Wang, H., Perry, J.W., Lauring, A.S., Neddermann, P., De Francesco, R. and Tai, A.W:
Gastroenteerology, ***146****, 1373-1385 (2014)*

Background & Aims
Positive-sense RNA viruses remodel intracellular membranes to generate specialized membrane compartments for viral replication. Several RNA viruses, including poliovirus and hepatitis C virus (HCV), require phosphatidylinositol (PI) 4-kinases for their replication. However, it is not known how PI 4-kinases and their product, PI(4)P, facilitate host membrane reorganization and viral replication. In addition, although the HCV replication compartment, known as the membranous web, is believed to be cholesterol enriched, the mechanisms by which this occurs have not been elucidated. We aimed to identify and characterize a PI 4-kinase effector in HCV replication.
Methods
We used a combination of microscopic and biochemical methods to study HCV replication, web morphology, the distribution of intracellular protein and PI(4)P, along with cholesterol trafficking in HCV-infected cells. PI 4-kinase and oxysterol-binding protein (OSBP) were inhibited using RNA interference or small molecules in cells expressing a full-length genotype 1b replicon or infected with the JFH-1 strain of HCV.
Results
OSBP was required for HCV replication and membranous web integrity. OSBP was recruited to membranous webs in a PI 4-kinase–dependent manner, and both these factors were found to regulate cholesterol trafficking to the web. We also found OSBP to be required for poliovirus infection but dispensable for dengue virus.
Conclusions
OSBP is a PI 4-kinase effector in HCV infection, and contributes to the integrity and cholesterol enrichment of the membranous web. OSBP might also be a PI 4-kinase effector in poliovirus infection and could be involved in replication of other viruses that require PI 4-kinases.

3.2181 Polymer–Peptide Delivery Platforms: Effect of Oligopeptide Orientation on Polymer-Based DNA Delivery

Parelkar, S.S., Letteri, r., Chan-Seng, D., Zolochevska, O., Ellis, J., Figueiredo, M. and Emrick, T.
Biomacromolecules, ***15(4)****, 1328-1336 (2014)*

The success of nonviral transfection using polymers hinges on efficient nuclear uptake of nucleic acid cargo and overcoming intra- and extracellular barriers. By incorporating PKKKRKV heptapeptide pendent groups as nuclear localization signals (NLS) on a polymer backbone, we demonstrate protein expression levels higher than those obtained from JetPEI and Lipofectamine 2000, the latter being notorious for coupling high transfection efficiency with cytotoxicity. The orientation of the NLS peptide grafts markedly affected transfection performance. Polymers with the sequence attached to the backbone from the valine residue achieved a level of nuclear translocation higher than the levels of those having the NLS groups attached in the opposite orientation. The differences in nuclear localization and DNA complexation strength between the two orientations correlated with a striking difference in protein expression, both in cell culture and *in vivo*. Polyplexes formed from these comb polymer structures exhibited transfection efficiencies superior to those of Lipofectamine 2000 but with greatly reduced toxicity. Moreover, these novel polymers, when administered by intramuscular ultrasound-mediated delivery, allowed a high level of reporter gene expression in mice, demonstrating their therapeutic promise *in vivo*.

3.2182 Deficiency of Sphingosine-1-phosphate Lyase Impairs Lysosomal Metabolism of the Amyloid Precursor Protein

Karaca, I., Tamboli, I.Y., Glebov, K., Richter, J., Fell, L.H., Grimm, M.O., Haupenthal, V.J., hartmann, T., Gräler, M.H., van Echten-Deckert, G. and Walter, J.
J. Biol. Chem., ***289(24)***, *16761-16772 (2014)*

Progressive accumulation of the amyloid β protein in extracellular plaques is a neuropathological hallmark of Alzheimer disease. Amyloid β is generated during sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. In addition to the proteolytic processing by secretases, APP is also metabolized by lysosomal proteases. Here, we show that accumulation of intracellular sphingosine-1-phosphate (S1P) impairs the metabolism of APP. Cells lacking functional S1P-lyase, which degrades intracellular S1P, strongly accumulate full-length APP and its potentially amyloidogenic C-terminal fragments (CTFs) as compared with cells expressing the functional enzyme. By cell biological and biochemical methods, we demonstrate that intracellular inhibition of S1P-lyase impairs the degradation of APP and CTFs in lysosomal compartments and also decreases the activity of γ-secretase. Interestingly, the strong accumulation of APP and CTFs in S1P-lyase-deficient cells was reversed by selective mobilization of Ca^{2+} from the endoplasmic reticulum or lysosomes. Intracellular accumulation of S1P also impairs maturation of cathepsin D and degradation of Lamp-2, indicating a general impairment of lysosomal activity. Together, these data demonstrate that S1P-lyase plays a critical role in the regulation of lysosomal activity and the metabolism of APP.

3.2183 Quantitation of Physiological and Biochemical Barriers to siRNA Liver Delivery via Lipid Nanoparticle Platform

Xu, Y., Keough, E., Roberts, J., Koeplinger, K., Lyman, M., Fauty, S., Carlini, E., Stern, M., Zhang, R., Yeh, S., Mahan, E., Wang, Y., Slaughter, D., Gindy, M., Raab, C., Thompson, C. and Hochman, J.
Mol. Pharmaceutics, ***11(5)***, *1424-1434 (2014)*

Effective delivery of small interfering RNA (siRNA) requires efficient cellular uptake and release into cytosol where it forms an active complex with RNAi induced silencing complex (RISC). Despite rapid developments in RNAi therapeutics, improvements in delivery efficiency of siRNA are needed to realize the full potential of this modality in broad therapeutic applications. We evaluated potential physiological and biochemical barrier(s) to the effective liver delivery of siRNA formulated in lipid nanoparticle (LNP) delivery vehicles. The comparative siRNA delivery performance of three LNPs was investigated in rats. They were assembled with either C14- or C18-anchored PEG-lipid(s), cationic lipid(s), and various helper lipid(s) and contained the same siRNA duplex. These LNPs demonstrated differentiated potency with ED_{50}'s ranging from 0.02 to 0.25 mg/kg. The two C14-PEG-LNPs had comparable siRNA exposure in plasma and liver, while the C18-PEG-LNP demonstrated a higher plasma siRNA exposure and a slower but sustained liver uptake. RISC bound siRNA within the liver, a more proximal measure of the pharmacologically active siRNA species, displayed loading kinetics that paralleled the target mRNA knockdown profile, with greater RISC loading associated with more potent LNPs. Liver perfusion and hepatocyte isolation experiments were performed following treatment of rats with LNPs containing VivoTag-fluorescently labeled siRNA. One hour after dosing a majority of the siRNA within the liver was associated with hepatocytes and was internalized (within small subcellular vesicles) with no significant cell surface association, indicating good liver tissue penetration, hepatocellular distribution, and internalization. Comparison of siRNA amounts in hepatocytes and subcellular fractions of the three LNPs suggests that endosomal escape is a significant barrier to siRNA delivery where cationic lipid seems to have a great impact. Quantitation of Ago-2 associated siRNA revealed that after endosomal escape further loss of siRNA occurs prior to RISC loading. This quantitative assessment of LNP-mediated siRNA delivery has highlighted potential barriers with respect to endosomal escape and incomplete RISC loading for delivery optimization efforts.

3.2184 Nongenomic Thyroid Hormone Signaling Occurs Through a Plasma Membrane–Localized Receptor

Kalyanaraman, H., Schwappacher, r., Joshua, J., Zhuang, S., Scott, B.T., Klos, M., Casteel, D.E., Frangos, J.A., Dillmann, W., Boss, G.R. and Pilz, R.B.
Science Signaling, ***7(326),*** *ra48 (2014)*

Thyroid hormone (TH) is essential for vertebrate development and the homeostasis of most adult tissues, including bone. TH stimulates target gene expression through the nuclear thyroid receptors TRα and TRβ; however, TH also has rapid, transcription-independent (nongenomic) effects. We found a previously uncharacterized plasma membrane–bound receptor that was necessary and sufficient for nongenomic TH

signaling in several cell types. We determined that this receptor is generated by translation initiation from an internal methionine of TRα, which produces a transcriptionally incompetent protein that is palmitoylated and associates with caveolin-containing plasma membrane domains. TH signaling through this receptor stimulated a pro-proliferative and pro-survival program by increasing the intracellular concentrations of calcium, nitric oxide (NO), and cyclic guanosine monophosphate (cGMP), which led to the sequential activation of protein kinase G II (PKGII), the tyrosine kinase Src, and extracellular signal–regulated kinase (ERK) and Akt signaling. Hypothyroid mice exhibited a cGMP-deficient state with impaired bone formation and increased apoptosis of osteocytes, which was rescued by a direct stimulator of guanylate cyclase. Our results link nongenomic TH signaling to a previously uncharacterized membrane-bound receptor, and identify NO synthase, guanylate cyclase, and PKGII as TH effectors that activate kinase cascades to regulate cell survival and proliferation.

3.2185 Sialidase NEU3 Dynamically Associates to Different Membrane Domains Specifically Modifying Their Ganglioside Pattern and Triggering Akt Phosphorylation

Bonardi, D., Papini, N., Pasini, M., Dileo, L., Orizio, F., Monti, E., Caimi, L., Venerando, B. and Bresciani, R.

PloS One, ***9(6)***, *e99405 (2014)*

Lipid rafts are known to regulate several membrane functions such as signaling, trafficking and cellular adhesion. The local enrichment in sphingolipids and cholesterol together with the low protein content allows their separation by density gradient flotation after extraction with non-ionic detergent at low temperature. These structures are also referred to as detergent resistant membranes (DRM). Among sphingolipids, gangliosides play important roles in different biological events, including signal transduction and tumorigenesis. Sialidase NEU3 shows high enzymatic specificity toward gangliosides. Moreover, the enzyme is present both at the cell surface and in endosomal structures and cofractionates with caveolin. Although changes in the expression level of NEU3 have been correlated to different tumors, little is known about the precise distribution of the protein and its ability in modifying the ganglioside composition of DRM and non-DRM, thus regulating intracellular events. By means of inducible expression cell system we found that i) newly synthesized NEU3 is initially associated to non-DRM; ii) at steady state the protein is equally distributed between the two membrane subcompartments, i.e., DRM and non-DRM; iii) NEU3 is degraded via the proteasomal pathway; iv) the enzyme specifically modifies the ganglioside composition of the membrane areas where it resides; and v) NEU3 triggers phosphorylation of Akt, even in absence of exogenously administered EGF. Taken together our data demonstrate that NEU3 regulates the DRM ganglioside content and it can be considered as a modulator of Akt phosphorylation, further supporting the role of this enzyme in cancer and tumorigenesis.

3.2186 P2X4 Forms Functional ATP-activated Cation Channels on Lysosomal Membranes Regulated by Luminal pH

Huang, P., Zou, Y., Zhong, X.Z., Cao, Q., Zhao, K., Zhu, M.X., Murrell-Lagnado, R and Dong, X-P.

J. Biol. Chem., ***289(25)***, *17658-17667 (2014)*

P2X receptors are commonly known as plasma membrane cation channels involved in a wide variety of cell functions. The properties of these channels have been extensively studied on the plasma membrane. However, studies in amoeba suggest that P2X receptors are also present intracellularly and involved in vesicle fusion with the plasma membrane. Recently, it was shown that in addition to plasma membrane expression, mammalian P2X4 was also localized intracellularly in lysosomes. However, it was not clear whether the lysosomal P2X4 receptors function as channels and how they are activated and regulated. In this paper, we show that both P2X4 and its natural ligand, ATP, are enriched in lysosomes of COS1 and HEK293 cells. By directly recording membrane currents from enlarged lysosomal vacuoles, we demonstrated that lysosomal P2X4 formed channels activated by ATP from the luminal side in a pH-dependent manner. While the acidic pH at the luminal side inhibited P2X4 activity, increasing the luminal pH in the presence of ATP caused P2X4 activation. We further showed that, as for the plasma membrane P2X4, the lysosomal P2X4 was potentiated by ivermectin but insensitive to suramin and PPADS, and it permeated the large cation *N*-methyl-d-glucamine upon activation. Our data suggest that P2X4 forms functional ATP-activated cation channels on lysosomal membranes regulated by luminal pH. Together with the reported fusion effect of intracellular P2X in lower organisms, we speculate that the lysosome-localized P2X4 may play specific roles in membrane trafficking of acidic organelles in mammalian cells.

3.2187 GRP78 is a novel receptor initiating a vascular barrier protective response to oxidized phospholipids
Birukova, A.A., Singleton, P.A., Gawlak, G., Tian, X., Mirzapoiazova, T., Mambetsariev, B., Dubrovskyi, O., Oskolkova, O.V., Bochkov, V.N. and Birukov, K.G.
*Mol. Biol. Cell, **25**, 2006-2016 (2014)*

Vascular integrity and the maintenance of blood vessel continuity are fundamental features of the circulatory system maintained through endothelial cell–cell junctions. Defects in the endothelial barrier become an initiating factor in several pathologies, including ischemia/reperfusion, tumor angiogenesis, pulmonary edema, sepsis, and acute lung injury. Better understanding of mechanisms stimulating endothelial barrier enhancement may provide novel therapeutic strategies. We previously reported that oxidized phospholipids (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [OxPAPC]) promote endothelial cell (EC) barrier enhancement both in vitro and in vivo. This study examines the initiating mechanistic events triggered by OxPAPC to increase vascular integrity. Our data demonstrate that OxPAPC directly binds the cell membrane–localized chaperone protein, GRP78, associated with its cofactor, HTJ-1. OxPAPC binding to plasma membrane–localized GRP78 leads to GRP78 trafficking to caveolin-enriched microdomains (CEMs) on the cell surface and consequent activation of sphingosine 1-phosphate receptor 1, Src and Fyn tyrosine kinases, and Rac1 GTPase, processes essential for cytoskeletal reorganization and EC barrier enhancement. Using animal models of acute lung injury with vascular hyperpermeability, we observed that HTJ-1 knockdown blocked OxPAPC protection from interleukin-6 and ventilator-induced lung injury. Our data indicate for the first time an essential role of GRP78 and HTJ-1 in OxPAPC-mediated CEM dynamics and enhancement of vascular integrity.

3.2188 Rab11 Regulates Trafficking of Trans-sialidase to the Plasma Membrane through the Contractile Vacuole Complex of Trypanosoma cruzi
Niyogi, S., Mucci, J., Campetella, O. and Docompo, R.
*PloS Pathogens, **10(6)**, e1004224 (2014)*

Trypanosoma cruzi is the etiologic agent of Chagas disease. Although this is not a free-living organism it has conserved a contractile vacuole complex (CVC) to regulate its osmolarity. This obligate intracellular pathogen is, in addition, dependent on surface proteins to invade its hosts. Here we used a combination of genetic and biochemical approaches to delineate the contribution of the CVC to the traffic of glycosylphosphatidylinositol (GPI)-anchored proteins to the plasma membrane of the parasite and promote host invasion. While *T. cruzi* Rab11 (GFP-TcRab11) localized to the CVC, a dominant negative (DN) mutant tagged with GFP (GFP-TcRab11DN) localized to the cytosol, and epimastigotes expressing this mutant were less responsive to hyposmotic and hyperosmotic stress. Mutant parasites were still able to differentiate into metacyclic forms and infect host cells. GPI-anchored *trans*-sialidase (TcTS), mucins of the 60–200 KDa family, and trypomastigote small surface antigen (TcTSSA II) co-localized with GFP-TcRab11 to the CVC during transformation of intracellular amastigotes into trypomastigotes. Mucins of the gp35/50 family also co-localized with the CVC during metacyclogenesis. Parasites expressing GFP-TcRab11DN prevented TcTS, but not other membrane proteins, from reaching the plasma membrane, and were less infective as compared to wild type cells. Incubation of these mutants in the presence of exogenous recombinant active, but not inactive, TcTS, and a sialic acid donor, before infecting host cells, partially rescued infectivity of trypomastigotes. Taking together these results reveal roles of TcRab11 in osmoregulation and trafficking of *trans*-sialidase to the plasma membrane, the role of *trans*-sialidase in promoting infection, and a novel unconventional mechanism of GPI-anchored protein secretion.

3.2189 Proteins of the Ciliary Axoneme Are Found on Cytoplasmic Membrane Vesicles during Growth of Cilia
Wood, C.R. and Rosenbaum, J.L.
*Current Biology, **24**, 1114-1120 (2014)*

The cilium is a specialized extension of the cell in which many specific proteins are admitted and retained, while many others are excluded or expelled. In order to maintain the organelle, the cell must possess mechanisms for the selective gating of protein entry, as well as for the targeted transport of proteins to the cilium from their sites of synthesis within the cell [1, 2, 3 and 4]. We hypothesized that the cell employs cytoplasmic vesicles as vehicles not only for the transport of proteins destined for the ciliary membrane but also for the transport of axonemal proteins to the cilium by means of peripheral association with vesicles. To test this hypothesis, we employed two different experimental strategies: (1) isolation and biochemical characterization of cytoplasmic vesicles that carry ciliary proteins, and (2) in situ localization of ciliary proteins on cytoplasmic vesicle surfaces using gold labeling and electron microscopy. Our findings

indicate that structural proteins destined for the ciliary axoneme are attached to the outer surfaces of cytoplasmic vesicles that carry integral ciliary membrane proteins during the process of ciliary growth.

3.2190 WLS Retrograde Transport to the Endoplasmic Reticulum during Wnt Secretion
Yu, J., Chia, J., Canning, C.A., Jones, C.M., Bard, F.A. and Virshup, D.M.
*Developmental Cell, **29**, 277-291 (2014)*

Wnts are transported to the cell surface by the integral membrane protein WLS (also known as Wntless, Evi, and GPR177). Previous studies of WLS trafficking have emphasized WLS movement from the Golgi to the plasma membrane (PM) and then back to the Golgi via retromer-mediated endocytic recycling. We find that endogenous WLS binds Wnts in the endoplasmic reticulum (ER), cycles to the PM, and then returns to the ER through the Golgi. We identify an ER-targeting sequence at the carboxyl terminus of native WLS that is critical for ER retrograde recycling and contributes to Wnt secretory function. Golgi-to-ER recycling of WLS requires the COPI regulator ARF as well as ERGIC2, an ER-Golgi intermediate compartment protein that is also required for the retrograde trafficking of the KDEL receptor and certain toxins. ERGIC2 is required for efficient Wnt secretion. ER retrieval is an integral part of the WLS transport cycle.

3.2191 Visualizing active membrane protein complexes by electron cryotomography
Gold, V.A.M., Leva, R., Walter, A., Pfanner, N., van der Laan, M. and Kühlbrandt, W.
*Nature Communications, **5**:4129 (2014)*

Unravelling the structural organization of membrane protein machines in their active state and native lipid environment is a major challenge in modern cell biology research. Here we develop the STAMP (Specifically TArgeted Membrane nanoParticle) technique as a strategy to localize protein complexes *in situ* by electron cryotomography (cryo-ET). STAMP selects active membrane protein complexes and marks them with quantum dots. Taking advantage of new electron detector technology that is currently revolutionizing cryotomography in terms of achievable resolution, this approach enables us to visualize the three-dimensional distribution and organization of protein import sites in mitochondria. We show that import sites cluster together in the vicinity of crista membranes, and we reveal unique details of the mitochondrial protein import machinery in action. STAMP can be used as a tool for site-specific labelling of a multitude of membrane proteins by cryo-ET in the future.

3.2192 Regulation of Nuclear Translocation of the Myb1 Transcription Factor by TvCyclophilin 1 in the Protozoan Parasite Trichomonas vaginalis
Hsu, H-M., Chu, C-H., Wang, Y-T., Lee, Y., Wei, S-Y., Liu, H-W., Ong, S-J., Chen, C. and Tai, J-H.
*J. Biol. Chem., **289(27)**, 19120-19136 (2014)*

In *Trichomonas vaginalis*, a Myb1 protein was previously demonstrated to repress transcription of an iron-inducible *ap65-1* gene. In this study, a human cyclophilin A homologue, *Tv*Cyclophilin 1 (*Tv*CyP1), was identified as a Myb1-binding protein using a bacterial two-hybrid library screening system. The recombinant *Tv*CyP1 exhibited typical peptidyl-prolyl isomerase activity with k_{cat}/K_m of ~7.1 μm^{-1} s^{-1}. In a pulldown assay, the His-tagged Myb1 interacted with a GST-*Tv*CyP1 fusion protein, which had an enzymatic proficiency half that of recombinant *Tv*CyP1. Both the enzymatic proficiency of GST-*Tv*CyP1 and its binding to His-Myb1 were eliminated by mutation of Arg^{63} in the catalytic motif or inhibited by cyclosporin A. *Tv*CyP1 was primarily localized to the hydrogenosomes by immunofluorescence assay, but it was also co-purified with Myb1 in certain vesicle fractions from differential and gradient centrifugations. Transgenic cells overexpressing HA-*Tv*CyP1 had a higher level of nuclear Myb1 but a much lower level of Myb1 associated with the vesicles than control and those overexpressing HA-*Tv*CyP1(R63A). Myb1 was detected at a much higher level in the HA-*Tv*CyP1 protein complex than in the HA-*Tv*CyP1(R63A) protein complex immunoprecipitated from P15 and P100, but not S100, fractions of postnuclear lysates. A *Tv*CyP1-binding motif, ^{105}YGPKWNK^{111}, was identified in Myb1 in which Gly^{106} and Pro^{107} were essential for its binding to *Tv*CyP1. Mutation of Gly^{106} and Pro^{107}, respectively, in HA-Myb1 resulted in cytoplasmic retention and elevated nuclear translocation of the overexpressed protein. These results suggest that *Tv*CyP1 may induce the release of Myb1 that is restrained to certain cytoplasmic vesicles prior to its nuclear translocation.

3.2193 The Lateral Membrane Organization and Dynamics of Myelin Proteins PLP and MBP Are Dictated by Distinct Galactolipids and the Extracellular Matrix
Ozgen, H., Schrimpf, W., Hendrix, J., de Jonge, J.C., Lamb, D.C., Hoekstra, D., Kahya, N. and baron, W.

*PloS One, **9(7)**, e101834 (2014)*

In the central nervous system, lipid-protein interactions are pivotal for myelin maintenance, as these interactions regulate protein transport to the myelin membrane as well as the molecular organization within the sheath. To improve our understanding of the fundamental properties of myelin, we focused here on the lateral membrane organization and dynamics of peripheral membrane protein 18.5-kDa myelin basic protein (MBP) and transmembrane protein proteolipid protein (PLP) as a function of the typical myelin lipids galactosylceramide (GalC), and sulfatide, and exogenous factors such as the extracellular matrix proteins laminin-2 and fibronectin, employing an oligodendrocyte cell line, selectively expressing the desired galactolipids. The dynamics of MBP were monitored by *z*-scan point fluorescence correlation spectroscopy (FCS) and raster image correlation spectroscopy (RICS), while PLP dynamics in living cells were investigated by circular scanning FCS. The data revealed that on an inert substrate the diffusion rate of 18.5-kDa MBP increased in GalC-expressing cells, while the diffusion coefficient of PLP was decreased in sulfatide-containing cells. Similarly, when cells were grown on myelination-promoting laminin-2, the lateral diffusion coefficient of PLP was decreased in sulfatide-containing cells. In contrast, PLP's diffusion rate increased substantially when these cells were grown on myelination-inhibiting fibronectin. Additional biochemical analyses revealed that the observed differences in lateral diffusion coefficients of both proteins can be explained by differences in their biophysical, i.e., galactolipid environment, specifically with regard to their association with lipid rafts. Given the persistence of pathological fibronectin aggregates in multiple sclerosis lesions, this fundamental insight into the nature and dynamics of lipid-protein interactions will be instrumental in developing myelin regenerative strategies.

3.2194 Ca2 +–CaM regulating viability of Candida guilliermondii under oxidative stress by acting on detergent resistant membrane proteins
An, B., Chen, Y., Li, B., Qin, G. and Tian, S.
*J. Proteomics, **109**, 38-49 (2014)*

Reactive oxygen species (ROS) play a vital role in reducing viability of yeast cells. The Ca^{2+}-CaM signaling pathways are involved in regulating the intracellular ROS level in yeast cells under stress. Detergent resistant membranes (DRMs), the sterol-rich microdomains, participate in a wide range of cellular processes including growth, trafficking and death in yeast cells. In the present study, we found that Trifluoperazine (TFP), an antagonist of CaM, could increase the viability of *Candida guilliermondii* cells under H_2O_2 stress. Based on comparative analysis of DRM sub proteomics, a total number of 29 differentially expressed protein spots were identified, among which 8 protein spots belong to the electron transport chain and 7 protein spots belong to transporters. It is suggested that TFP treatment could modulate the intracellular ROS generation in yeast cells. We additionally ascertained that TFP treatment could effectively alleviate the ROS accumulation and protein damage in *C. guilliermondii* cells under H_2O_2 stress, via investigating the intracellular ROS levels and protein oxidative damage in yeast cells. These findings firstly revealed that the Ca^{2+}-CaM signaling pathway is related to the viability of yeast cells under H_2O_2 stress, and provide novel evidences for exploring Ca^{2+}-CaM's role in regulating this viability via acting on DRM proteins.

3.2195 Comparing the different morphotypes of a fish pathogen - implications for key virulence factors in Flavobacterium columnare
Laanto, E., Penttinen, R.K., Bamford, J.K.H. and Sundberg, L-R.
*BMC Microbiol., **14**:170 (2014)*

Background
Flavobacterium columnare (Bacteroidetes) is the causative agent of columnaris disease in farmed freshwater fish around the world. The bacterium forms three colony morphotypes (Rhizoid, Rough and Soft), but the differences of the morphotypes are poorly known. We studied the virulence of the morphotypes produced by *F. columnare* strain B067 in rainbow trout (*Onconrhynchus mykiss*) and used high-resolution scanning electron microscopy to identify the fine structures of the cells grown in liquid and on agar. We also analysed the proteins secreted extracellularly and in membrane vesicles to identify possible virulence factors.
Results
Only the Rhizoid morphotype was virulent in rainbow trout. Under electron microscopy, the cells of Rhizoid and Soft morphotypes were observed to display an organised structure within the colony, whereas in the Rough type this internal organisation was absent. Planktonic cells of the Rhizoid and Rough morphotypes produced large membrane vesicles that were not seen on the cells of the Soft morphotype.

The vesicles were purified and analysed. Two proteins with predicted functions were identified, OmpA and SprF. Furthermore, the Rhizoid morphotype secreted a notable amount of a small, unidentified 13 kDa protein absent in the Rough and Soft morphotypes, indicating an association with bacterial virulence.
Conclusions
Our results suggest three factors that are associated with the virulence of *F. columnare*: the coordinated organisation of cells, a secreted protein and outer membrane vesicles. The internal organisation of the cells within a colony may be associated with bacterial gliding motility, which has been suggested to be connected with virulence in *F. columnare*. The function of the secreted 13 kDa protein by the cells of the virulent morphotype cells remains unknown. The membrane vesicles might be connected with the adhesion of cells to the surfaces and could also carry potential virulence factors. Indeed, OmpA is a virulence factor in several bacterial pathogens, often linked with adhesion and invasion, and SprF is a protein connected with gliding motility and the protein secretion of flavobacteria.

3.2196 Fluvoxamine alleviates ER stress via induction of Sigma-1 receptor
Omi, T., Tanimukai, H., Kanayama, D., Sakagami, Y., Tagami, S., Okochi, M., Morihara, T., Sato, M., Yanagida, K., Kitasyoji, A., Hara, H., Imaizumi, K., Maurice, T., Chevallier, N., marchal, S., Takeda, M. and Kudo, T.
*Cell Death and Disease, **5**, e1332 (2014)*

We recently demonstrated that endoplasmic reticulum (ER) stress induces sigma-1 receptor (Sig-1R) expression through the PERK pathway, which is one of the cell's responses to ER stress. In addition, it has been demonstrated that induction of Sig-1R can repress cell death signaling. Fluvoxamine (Flv) is a selective serotonin reuptake inhibitor (SSRI) with a high affinity for Sig-1R. In the present study, we show that treatment of neuroblastoma cells with Flv induces Sig-1R expression by increasing ATF4 translation directly, through its own activation, without involvement of the PERK pathway. The Flv-mediated induction of Sig-1R prevents neuronal cell death resulting from ER stress. Moreover, Flv-induced ER stress resistance reduces the infarct area in mice after focal cerebral ischemia. Thus, Flv, which is used frequently in clinical practice, can alleviate ER stress. This suggests that Flv could be a feasible therapy for cerebral diseases caused by ER stress.

3.2197 The carrying pigeons of the cell: exosomes and their role in infectious diseases caused by human pathogens
Fleming, A., Sampey, G., Chung, M-C., Bailey, C., van Hoek, M.L., Kashanchi, F. and Hakami, R.M.
*Pathogens and Disease, **71(2)**, 107-118 (2014)*

Exosomes have recently been classified as the newest family members of 'bioactive vesicles' that function to promote intercellular communication. Long ignored and thought to be only a mechanism by which cellular waste is removed, exosomes have garnered a huge amount of interest in recent years as their critical functions in maintaining homeostasis through intercellular communication and also in different types of diseases have been demonstrated. Many groundbreaking studies of exosome functions have been performed in the cancer field and the infectious disease areas of study, revealing the importance and also the fascinating complexity of exosomal packaging, targeting, and functions. Selective packaging of exosomes in response to the type of infection, exosomal modulation of the immune response and host signaling pathways, exosomal regulation of pathogen spread, and effects of exosomes on the degree of pathogenesis have all been well documented. In this review, we provide a synthesis of the current understanding of the role of exosomes during infections caused by human pathogens and discuss the implications of these findings for a better understanding of pathogenic mechanisms and future therapeutic and diagnostic applications.

3.2198 Extracellular vesicles produced by the Gram-positive bacterium Bacillus subtilis are disrupted by the lipopeptide surfactin
Brown, L., Kessler, a., Cabezas-Sanchez, P. Luque-garcia, J.L. and casadevall, A.
*Mol. Microbiol., **93(1)**, 183-198 (2014)*

Previously, extracellular vesicle production in Gram-positive bacteria was dismissed due to the absence of an outer membrane, where Gram-negative vesicles originate, and the difficulty in envisioning how such a process could occur through the cell wall. However, recent work has shown that Gram-positive bacteria produce extracellular vesicles and that the vesicles are biologically active. In this study, we show that *Bacillus subtilis* produces extracellular vesicles similar in size and morphology to other bacteria, characterized vesicles using a variety of techniques, provide evidence that these vesicles are actively

produced by cells, show differences in vesicle production between strains, and identified a mechanism for such differences based on vesicle disruption. We found that in wild strains of *B. subtilis*, surfactin disrupted vesicles while in laboratory strains harbouring a mutation in the gene *sfp*, vesicles accumulated in the culture supernatant. Surfactin not only lysed *B. subtilis* vesicles, but also vesicles from *Bacillus anthracis*, indicating a mechanism that crossed species boundaries. To our knowledge, this is the first time a gene and a mechanism has been identified in the active disruption of extracellular vesicles and subsequent release of vesicular cargo in Gram-positive bacteria. We also identify a new mechanism of action for surfactin.

3.2199 Integrating mitosis, toxicity, and transgene expression in a telecommunications packet-switched network model of lipoplex-mediated gene delivery
Martin, T.M., Wysocki, B.J., Beyersdorf, J.P., Wysocki, T.A. and pannier, A.K.
Biotechnol. Bioeng., ***111(8)***, *1659-1671 (2014)*

Gene delivery systems transport exogenous genetic information to cells or biological systems with the potential to directly alter endogenous gene expression and behavior with applications in functional genomics, tissue engineering, medical devices, and gene therapy. Nonviral systems offer advantages over viral systems because of their low immunogenicity, inexpensive synthesis, and easy modification but suffer from lower transfection levels. The representation of gene transfer using models offers perspective and interpretation of complex cellular mechanisms, including nonviral gene delivery where exact mechanisms are unknown. Here, we introduce a novel telecommunications model of the nonviral gene delivery process in which the delivery of the gene to a cell is synonymous with delivery of a packet of information to a destination computer within a packet-switched computer network. Such a model uses nodes and layers to simplify the complexity of modeling the transfection process and to overcome several challenges of existing models. These challenges include a limited scope and limited time frame, which often does not incorporate biological effects known to affect transfection. The telecommunication model was constructed in MATLAB to model lipoplex delivery of the gene encoding the green fluorescent protein to HeLa cells. Mitosis and toxicity events were included in the model resulting in simulation outputs of nuclear internalization and transfection efficiency that correlated with experimental data. A priori predictions based on model sensitivity analysis suggest that increasing endosomal escape and decreasing lysosomal degradation, protein degradation, and GFP-induced toxicity can improve transfection efficiency by three-fold. Application of the telecommunications model to nonviral gene delivery offers insight into the development of new gene delivery systems with therapeutically relevant transfection levels

3.2200 Isolation and characterization of lipid rafts in Emiliania huxleyi: a role for membrane microdomains in host–virus interactions
Rose, S.L., Fulton, J.M., Brown, C.M., natale, f., Van Mooy, B.A.S. and Bidle, K.D.
Environmental Microbiol., ***16(4)***, *1150-1166 (2014)*

Coccolithoviruses employ a suite of glycosphingolipids (GSLs) to successfully infect the globally important coccolithophore *Emiliania huxleyi*. Lipid rafts, chemically distinct membrane lipid microdomains that are enriched in GSLs and are involved in sensing extracellular stimuli and activating signalling cascades through protein–protein interactions, likely play a fundamental role in host–virus interactions. Using combined lipidomics, proteomics and bioinformatics, we isolated and characterized the lipid and protein content of lipid rafts from control *E. huxleyi* cells and those infected with EhV86, the type strain for *Coccolithovirus*. Lipid raft-enriched fractions were isolated and purified as buoyant, detergent-resistant membranes (DRMs) in OptiPrep density gradients. Transmission electron microscopy of vesicle morphology, polymerase chain reaction amplification of the EhV major capsid protein gene and immunoreactivity to flotillin antisera served as respective physical, molecular and biochemical markers. Subsequent lipid characterization of DRMs via high performance liquid chromatography-triple quadrapole mass spectrometry revealed four distinct GSL classes. Parallel proteomic analysis confirmed flotillin as a major lipid raft protein, along with a variety of proteins affiliated with host defence, programmed cell death and innate immunity pathways. The detection of an EhV86-encoded C-type lectin-containing protein confirmed that infection occurs at the interface between lipid rafts and cellular stress/death pathways via specific GSLs and raft-associated proteins.

3.2201 Fractionation of Subcellular Membrane Vesicles of Epithelial and Non-epithelial Cells by OptiPrep™ Density Gradient Ultracentrifugation
Li, X. and donowitz, M.
Methods in Mol. Biol., ***1174***, *85-99 (2014)*

Density gradient ultracentrifugation (DGUC) is widely used for physical isolation (enrichment rather than purification) of subcellular membrane vesicles. It has been a valuable tool to study specific subcellular localization and dynamic trafficking of proteins. While sucrose has been the main component of density gradients, several years ago, synthetic OptiPrep™ (iodixanol) began being used for separation of organelles due to its iso-osmotic property. Here, we describe a detailed protocol for density gradient fractionation of various mammalian subcellular vesicles, including endoplasmic reticulum (ER), Golgi apparatus, endosomes, and lipid rafts, as well as apical and basolateral membranes of polarized epithelial cells.

3.2202 **A Foundation for Reliable Spatial Proteomics Data Analysis**
Gatto, L., Breckels, L.M., Burger, T., Nightingale, D.J.H., Groen, A., Campbell,. C., Nikolovski, N., Mulvey, C.M., Christoforout, A., Ferro, M. and Lilley, K.S.
*Mol. Cell Proteomics, **13(8)**, 1937-1952 (2014)*

Quantitative mass-spectrometry-based spatial proteomics involves elaborate, expensive, and time-consuming experimental procedures, and considerable effort is invested in the generation of such data. Multiple research groups have described a variety of approaches for establishing high-quality proteome-wide datasets. However, data analysis is as critical as data production for reliable and insightful biological interpretation, and no consistent and robust solutions have been offered to the community so far. Here, we introduce the requirements for rigorous spatial proteomics data analysis, as well as the statistical machine learning methodologies needed to address them, including supervised and semi-supervised machine learning, clustering, and novelty detection. We present freely available software solutions that implement innovative state-of-the-art analysis pipelines and illustrate the use of these tools through several case studies involving multiple organisms, experimental designs, mass spectrometry platforms, and quantitation techniques. We also propose sound analysis strategies for identifying dynamic changes in subcellular localization by comparing and contrasting data describing different biological conditions. We conclude by discussing future needs and developments in spatial proteomics data analysis.

3.2203

3.2204 **Signaling components of the 1α,25(OH)2D3-dependent Pdia3 receptor complex are required for Wnt5a calcium-dependent signaling**
Doroudi, M., Olivares-Navarrete, R., Hyzy, S., Boyan, B. and Schwartz, Z.
*Biochim. Biophys. Acta, **1843**, 2365-2375 (2014)*

Wnt5a and 1 α,25$(OH)_2D_3$ are important regulators of endochondral ossification. In osteoblasts and growth plate chondrocytes, 1 α,25$(OH)_2D_3$ initiates rapid effects via its membrane-associated receptor protein disulfide isomerase A3 (Pdia3) in caveolae, activating phospholipase A_2 (PLA_2)-activating protein (PLAA), calcium/calmodulin-dependent protein kinase II (CaMKII), and PLA_2, resulting in protein kinase C (PKC) activation. Wnt5a initiates its calcium-dependent effects via intracellular calcium release, activating PKC and CaMKII. We investigated the requirement for components of the Pdia3 receptor complex in Wnt5a calcium-dependent signaling. We determined that Wnt5a signals through a CaMKII/PLA_2/PGE_2/PKC cascade. Silencing or blocking Pdia3, PLAA, or vitamin D receptor (VDR), and inhibition of calmodulin (CaM), CaMKII, or PLA_2 inhibited Wnt5a-induced PKC activity. Wnt5a activated PKC in caveolin-1-silenced cells, but methyl-beta-cyclodextrin reduced its stimulatory effect. 1 α,25$(OH)_2D_3$ reduced stimulatory effects of Wnt5a on PKC in a dose-dependent manner. In contrast, Wnt5a had a biphasic effect on 1 α,25$(OH)_2D_3$-stimulated PKC activation; 50 ng/ml Wnt5a caused a 2-fold increase in 1 α,25$(OH)_2D_3$-stimulated PKC but higher Wnt5a concentrations reduced 1 α,25$(OH)_2D_3$-stimulated PKC activation. Western blots showed that Wnt receptors Frizzled2 (FZD2) and Frizzled5 (FZD5), and receptor tyrosine kinase-like orphan receptor 2 (ROR2) were localized to caveolae. Blocking ROR2, but not FZD2 or FZD5, abolished the stimulatory effects of 1 α,25$(OH)_2D_3$ on PKC and CaMKII. 1 α,25$(OH)_2D_3$ membrane receptor complex components (Pdia3, PLAA, caveolin-1, CaM) interacted with Wnt5a receptors/co-receptors (ROR2, FZD2, FZD5) in immunoprecipitation studies, interactions that changed with either 1 α,25$(OH)_2D_3$ or Wnt5a treatment. This study demonstrates that 1 α,25$(OH)_2D_3$ and Wnt5a mediate their effects via similar receptor components and suggests that these pathways may interact.

3.2205 The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of Arabidopsis thaliana

Busse-Wicher, M., Gomes, T.C., Tryfona, T., Nikolovski, N., Stott, K., Grantham, N.J., Bolam, D.N., Skaf, M.S. and Dupree, P.

Plant J., ***79***, *492-506 (2014)*

The interaction between xylan and cellulose microfibrils is important for secondary cell wall properties in vascular plants; however, the molecular arrangement of xylan in the cell wall and the nature of the molecular bonding between the polysaccharides are unknown. In dicots, the xylan backbone of β-(1,4)-linked xylosyl residues is decorated by occasional glucuronic acid, and approximately one-half of the xylosyl residues are O-acetylated at C-2 or C-3. We recently proposed that the even, periodic spacing of GlcA residues in the major domain of dicot xylan might allow the xylan backbone to fold as a twofold helical screw to facilitate alignment along, and stable interaction with, cellulose fibrils; however, such an interaction might be adversely impacted by random acetylation of the xylan backbone. Here, we investigated the arrangement of acetyl residues in Arabidopsis xylan using mass spectrometry and NMR. Alternate xylosyl residues along the backbone are acetylated. Using molecular dynamics simulation, we found that a twofold helical screw conformation of xylan is stable in interactions with both hydrophilic and hydrophobic cellulose faces. Tight docking of xylan on the hydrophilic faces is feasible only for xylan decorated on alternate residues and folded as a twofold helical screw. The findings suggest an explanation for the importance of acetylation for xylan–cellulose interactions, and also have implications for our understanding of cell wall molecular architecture and properties, and biological degradation by pathogens and fungi. They will also impact strategies to improve lignocellulose processing for biorefining and bioenergy.

3.2206 Endoplasmic Reticulum-associated Degradation of Niemann-Pick C1: EVIDENCE FOR THE ROLE OF HEAT SHOCK PROTEINS AND IDENTIFICATION OF LYSINE RESIDUES THAT ACCEPT UBIQUITIN

Nakasone, N., Nakamura, Y.S., Higaki, K., Oumi, N., Ohno, K. and Ninomiya, H.

J. Biol. Chem., ***289(28)***, *19714-19725 (2014)*

Most cases with Niemann-Pick disease type C carry mutations in *NPC1*. Some of the mutations, including the most frequent I1061T, give rise to unstable proteins selected for endoplasmic reticulum-associated degradation. The purpose of the current study was to shed mechanistic insights into the degradation process. A proteasome inhibitor MG132 prolonged the life span of the wild-type NPC1 expressed in COS cells. The expressed protein associated with multiple chaperones including heat shock protein 90 (Hsp90), Hsp70, heat shock cognate protein 70 (Hsc70), and calnexin. Accordingly, expression of an E3 ligase CHIP (carboxyl terminus of Hsp70-interacting protein) enhanced MG132-induced accumulation of ubiquitylated NPC1. Co-expression and RNAi knockdown experiments in HEK cells indicated that Hsp70/Hsp90 stabilized NPC1, whereas Hsc70 destabilized it. In human fibroblasts carrying the I1061T mutation, adenovirus-mediated expression of Hsp70 or treatment with an HSP-inducer geranylgeranylacetone (GGA) increased the level of the mutant protein. In GGA-treated cells, the rescued protein was localized in the late endosome and ameliorated cholesterol accumulation. MALDI-TOF mass spectrometry revealed three lysine residues at amino acids 318, 792, and 1180 as potential ubiquitin-conjugation sites. Substitutions of the three residues with alanine yielded a mutant protein with a steady-state level more than three times higher than that of the wild-type. Introduction of the same substitutions to the I1061T mutant resulted in an increase in its protein level and functional restoration. These findings indicated the role of HSPs in quality control of NPC1 and revealed the role of three lysine residues as ubiquitin-conjugation sites.

3.2207 SLC17A9 Protein Functions as a Lysosomal ATP Transporter and Regulates Cell Viability

Cao, Q., Zhao, K., Zhong, X.Z., Zou, Y., Yu, H., Huang, P., Xu, T-L. and Dong, X-P.

J. Biol. Chem., ***289(33)***, *23189-23199 (2014)*

Lysosomes contain abundant ATP, which is released through lysosomal exocytosis following exposure to various stimuli. However, the molecular mechanisms underlying lysosomal ATP accumulation remain unknown. The vesicular nucleotide transporter, also known as solute carrier family 17 member 9 (SLC17A9), has been shown to function in ATP transport across secretory vesicles/granules membrane in adrenal chromaffin cells, T cells, and pancreatic cells. Here, using mammalian cell lines, we report that SLC17A9 is highly enriched in lysosomes and functions as an ATP transporter in those organelles. SLC17A9 deficiency reduced lysosome ATP accumulation and compromised lysosome function, resulting

in cell death. Our data suggest that SLC17A9 activity mediates lysosomal ATP accumulation and plays an important role in lysosomal physiology and cell viability.

3.2208 Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy
Schapansky, J., Nardozzi, J.D., Felizia, F. and LaVoie, M.J.
Hum. Mol. Genet., ***23(16)***, *4201-4214 (2014)*

Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common cause of familial and idiopathic Parkinson's disease. However, the mechanisms for activating its physiological function are not known, hindering identification of the biological role of endogenous LRRK2. The recent discovery that LRRK2 is highly expressed in cells of the innate immune system and genetic association is a risk factor for autoimmune disorders implies an important role for LRRK2 in pathology outside of the central nervous system. Thus, an examination of endogenous LRRK2 in immune cells could provide insight into the protein's function. Here, we establish that stimulation of specific Toll-like receptors results in a complex biochemical activation of endogenous LRRK2, with early phosphorylation of LRRK2 preceding its dimerization and membrane translocation. Membrane-associated LRRK2 co-localized to autophagosome membranes following either TLR4 stimulation or mTOR inhibition with rapamycin. Silencing of endogenous LRRK2 expression resulted in deficits in the induction of autophagy and clearance of a well-described macroautophagy substrate, demonstrating the critical role of endogenous LRRK2 in regulating autophagy. Inhibition of LRRK2 kinase activity also reduced autophagic degradation and suggested the importance of the kinase domain in the regulation of autophagy. Our results demonstrate a well-orchestrated series of biochemical events involved in the activation of LRRK2 important to its physiological function. With similarities observed across multiple cell types and stimuli, these findings are likely relevant in all cell types that natively express endogenous LRRK2, and provide insights into LRRK2 function and its role in human disease.

3.2209 A systems-level approach to understanding transcriptional regulation by p53 during mammalian hibernation
Pan, P., Treat, M.D. and van Breukelen, F.
J. Exp. Biol., ***217***, *2489-2498 (2014)*

Presumably to conserve energy, many mammals enter into hibernation during the winter. Homeostatic processes such as transcription and translation are virtually arrested. To further elucidate transcriptional regulation during hibernation, we studied the transcription factor p53. Here, we demonstrate that changes in liver mRNA and protein concentrations of known regulators of p53 are consistent with activation. p53 mRNA and protein concentrations are unrelated. Importantly, p53 protein concentration is increased ~2-fold during the interbout arousal that punctuates bouts of torpor. As a result, both the interbout arousal and the torpid state are characterized by high levels of nuclear-localized p53. Chromatin immunoprecipitation assays indicate that p53 binds DNA during the winter. Furthermore, p53 recruits RNA polymerase II, as indicated by nuclear run-on data. However, and consistent with previous data indicating an arrest of transcriptional elongation during torpor, p53 ‘activity’ does not result in expected changes in target gene transcripts. These data demonstrate the importance of using a systems level-approach in understanding a complex phenotype such as mammalian hibernation. Relying on interpretations of data that are based on steady-state regulation in other systems may be misleading in the context of non-steady-state conditions such as torpor.

3.2210 Nuclear RhoA signaling regulates MRTF-dependent SMC-specific transcription
Staus, D.P., Weise-Cross, L., magnum, K.D., Medlin, M.D., mangiante, L., Taylor, J.M. and Mack, C.P.
Am. J. Physiol. Heart Circ. Physiol., ***307***, *H379-H390 (2014)*

We have previously shown that RhoA-mediated actin polymerization stimulates smooth muscle cell (SMC)-specific transcription by regulating the nuclear localization of the myocardin-related transcription factors (MRTFs). On the basis of the recent demonstration that nuclear G-actin regulates MRTF nuclear export and observations from our laboratory and others that the RhoA effector, mDia2, shuttles between the nucleus and cytoplasm, we investigated whether nuclear RhoA signaling plays a role in regulating MRTF activity. We identified sequences that control mDia2 nuclear-cytoplasmic shuttling and used mDia2 variants to demonstrate that the ability of mDia2 to fully stimulate MRTF nuclear accumulation and SMC-specific gene transcription was dependent on its localization to the nucleus. To test whether RhoA signaling promotes nuclear actin polymerization, we established a fluorescence recovery after photobleaching (FRAP)-based assay to measure green fluorescent protein-actin diffusion in the nuclear

compartment. Nuclear actin FRAP was delayed in cells expressing nuclear-targeted constitutively active mDia1 and mDia2 variants and in cells treated with the polymerization inducer, jasplakinolide. In contrast, FRAP was enhanced in cells expressing a nuclear-targeted variant of mDia that inhibits both mDia1 and mDia2. Treatment of 10T1/2 cells with sphingosine 1-phosphate induced RhoA activity in the nucleus and forced nuclear localization of RhoA or the Rho-specific guanine nucleotide exchange factor (GEF), leukemia-associated RhoGEF, enhanced the ability of these proteins to stimulate MRTF activity. Taken together, these data support the emerging idea that RhoA-dependent nuclear actin polymerization has important effects on transcription and nuclear structure.

3.2211 Proteomic Analysis of the EWS-Fli-1 Interactome Reveals the Role of the Lysosome in EWS-Fli-1 Turnover

Elzi, D.J., Song, M., Hakala, K., Weintraub, S.T. and Shiio, Y.
J. Proteome Res., ***13(8)***, *3783-3791 (2014)*

Ewing sarcoma is a cancer of bone and soft tissue in children that is characterized by a chromosomal translocation involving EWS and an Ets family transcription factor, most commonly Fli-1. EWS-Fli-1 fusion accounts for 85% of cases. The growth and survival of Ewing sarcoma cells are critically dependent on EWS-Fli-1. A large body of evidence has established that EWS-Fli-1 functions as a DNA-binding transcription factor that regulates the expression of a number of genes important for cell proliferation and transformation. However, little is known about the biochemical properties of the EWS-Fli-1 protein. We undertook a series of proteomic analyses to dissect the EWS-Fli-1 interactome. Employing a proximity-dependent biotinylation technique, BioID, we identified cation-independent mannose 6-phosphate receptor (CIMPR) as a protein located in the vicinity of EWS-Fli-1 within a cell. CIMPR is a cargo that mediates the delivery of lysosomal hydrolases from the trans-Golgi network to the endosome, which are subsequently transferred to the lysosomes. Further molecular cell biological analyses uncovered a role for lysosomes in the turnover of the EWS-Fli-1 protein. We demonstrate that an mTORC1 active-site inhibitor, torin 1, which stimulates the TFEB-lysosome pathway, can induce the degradation of EWS-Fli-1, suggesting a potential therapeutic approach to target EWS-Fli-1 for degradation.

3.2212 Extracellular vesicles shed from gefitinib-resistant nonsmall cell lung cancer regulate the tumor microenvironment

Choi, D-Y., You, S., Jung, J.H., Lee, J.C., Rho, J.K., Lee, K.Y., Freeman, M.R., Kim, K.P. and Kim, J.
Proteomics, ***14(16)***, *1845-1856 (2014)*

Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), including gefitinib, are the first-line treatment of choice for nonsmall cell lung cancer patients who harbor activating EGFR mutations, however, acquired resistance to EGFR-TKIs is inevitable. The main objective of this study was to identify informative protein signatures of extracellular vesicles (EV) derived from gefitinib-resistant nonsmall cell lung cancer cells using proteomics analysis. Nano-LC–MS/MS analysis identified with high confidence (false discovery rate < 0.05, fold change ≥2) 664 EV proteins enriched in PC9R cells, which are resistant to gefitinib due to EGFR T790M mutation. Computational analyses suggested components of several signal transduction mechanisms including the AKT (also PKB, protein kinase B)/mTOR (mechanistic target of rapamycin) pathway are overrepresented in EV from PC9R cells. Treatment of recipient cells with EV harvested from PC9R cells increased phosphorylation of signaling molecules, and enhanced proliferation, invasion, and drug resistance to gefitinib-induced apoptosis. Dose- and time-dependent pharmaceutical inhibition of AKT/mTOR pathway overcame drug resistance of PC9R cells and those of H1975 exhibiting EGFR T790M mutation. Our findings provide new insight into an oncogenic EV protein signature regulating tumor microenvironment, and will aid in the development of novel diagnostic strategies for prediction and assessment of gefitinib resistance.

3.2213 Decoration of Outer Membrane Vesicles with Multiple Antigens by Using an Autotransporter Approach

Daleke-Schermerhorn, M.H: et al
Appl. Envir. Microbiol., ***80(18)***, *5854-5865 (2014)*

Outer membrane vesicles (OMVs) are spherical nanoparticles that naturally shed from Gram-negative bacteria. They are rich in immunostimulatory proteins and lipopolysaccharide but do not replicate, which increases their safety profile and renders them attractive vaccine vectors. By packaging foreign polypeptides in OMVs, specific immune responses can be raised toward heterologous antigens in the context of an intrinsic adjuvant. Antigens exposed at the vesicle surface have been suggested to elicit

protection superior to that from antigens concealed inside OMVs, but hitherto robust methods for targeting heterologous proteins to the OMV surface have been lacking. We have exploited our previously developed hemoglobin protease (Hbp) autotransporter platform for display of heterologous polypeptides at the OMV surface. One, two, or three of the Mycobacterium tuberculosis antigens ESAT6, Ag85B, and Rv2660c were targeted to the surface of Escherichia coli OMVs upon fusion to Hbp. Furthermore, a hypervesiculating Δ*tolR* Δ*tolA* derivative of attenuated Salmonella enterica serovar Typhimurium SL3261 was generated, enabling efficient release and purification of OMVs decorated with multiple heterologous antigens, exemplified by the M. tuberculosis antigens and epitopes from Chlamydia trachomatis major outer membrane protein (MOMP). Also, we showed that delivery of Salmonella OMVs displaying Ag85B to antigen-presenting cells *in vitro* results in processing and presentation of an epitope that is functionally recognized by Ag85B-specific T cell hybridomas. In conclusion, the Hbp platform mediates efficient display of (multiple) heterologous antigens, individually or combined within one molecule, at the surface of OMVs. Detection of antigen-specific immune responses upon vesicle-mediated delivery demonstrated the potential of our system for vaccine development.

3.2214 Extracellular membrane vesicles secreted by mycoplasma Acholeplasma laidlawii PG8 are enriched in virulence proteins

Chernov, V., Mouzykantov, A.A., baranova, N.B., Medvedeva, E.S., Grygorieva, T.Y., Trushin, M.V., Vishnyakov, I.E., Sabantsev, A.V., Borchsenius, S.N. and Chernova, O.A.
*J. Proteomics, **110**, 117-128 (2014)*

Mycoplasmas (class Mollicutes), the smallest prokaryotes capable of self-replication, as well as Archaea, Gram-positive and Gram-negative bacteria constitutively produce extracellular vesicles (EVs). However, little is known regarding the content and functions of mycoplasma vesicles. Here, we present for the first time a proteomics-based characterisation of extracellular membrane vesicles from *Acholeplasma laidlawii* PG8. The ubiquitous mycoplasma is widespread in nature, found in humans, animals and plants, and is the causative agent of phytomycoplasmoses and the predominant contaminant of cell cultures. Taking a proteomics approach using LC‑ESI-MS/MS, we identified 97 proteins. Analysis of the identified proteins indicated that *A. laidlawii*-derived EVs are enriched in virulence proteins that may play critical roles in mycoplasma-induced pathogenesis. Our data will help to elucidate the functions of mycoplasma-derived EVs and to develop effective methods to control infections and contaminations of cell cultures by mycoplasmas.
In the present study, we have documented for the first time the proteins in EVs secreted by mycoplasma vesicular proteins identified in this study are likely involved in the adaptation of bacteria to stressors, survival in microbial communities and pathogen‑host interactions. These findings suggest that the secretion of EVs is an evolutionally conserved and universal process that occurs in organisms from the simplest wall-less bacteria to complex organisms and indicate the necessity of developing new approaches to control infects.

3.2215 Outer Membrane Vesicles Mediate Transport of Biologically Active Vibrio cholerae Cytolysin (VCC) from V. cholerae Strains

Elluri, S., Enow, C., Vdovikova, S., Rompikuntal, P.K., Dongre, M., Carlsson, S., Pal, A., Uhlin, B.E. and Wai, S.N.
*PloS One, **9(9)**, e106731 (2014)*

Background
Outer membrane vesicles (OMVs) released from Gram-negative bacteria can serve as vehicles for the translocation of virulence factors. *Vibrio cholerae* produce OMVs but their putative role in translocation of effectors involved in pathogenesis has not been well elucidated. The *V. cholerae* cytolysin (VCC), is a pore-forming toxin that lyses target eukaryotic cells by forming transmembrane oligomeric β-barrel channels. It is considered a potent toxin that contributes to *V. cholerae* pathogenesis. The mechanisms involved in the secretion and delivery of the VCC have not been extensively studied.
Methodology/Principal Findings
OMVs from *V. cholerae* strains were isolated and purified using a differential centrifugation procedure and Optiprep centrifugation. The ultrastructure and the contents of OMVs were examined under the electron microscope and by immunoblot analyses respectively. We demonstrated that VCC from *V. cholerae* strain V:5/04 was secreted in association with OMVs and the release of VCC via OMVs is a common feature among *V. cholerae* strains. The biological activity of OMV-associated VCC was investigated using contact hemolytic assay and epithelial cell cytotoxicity test. It showed toxic activity on both red blood cells and epithelial cells. Our results indicate that the OMVs architecture might play a role in stability of VCC and

thereby can enhance its biological activities in comparison with the free secreted VCC. Furthermore, we tested the role of OMV-associated VCC in host cell autophagy signalling using confocal microscopy and immunoblot analysis. We observed that OMV-associated VCC triggered an autophagy response in the target cell and our findings demonstrated for the first time that autophagy may operate as a cellular defence mechanism against an OMV-associated bacterial virulence factor.
Conclusion/Significance
Biological assays of OMVs from the *V. cholerae* strain V:5/04 demonstrated that OMV-associated VCC is indeed biologically active and induces toxicity on mammalian cells and furthermore can induce autophagy.

3.2216 Exosomes from Human Immunodeficiency Virus Type 1 (HIV-1)-Infected Cells License Quiescent CD4+ T Lymphocytes To Replicate HIV-1 through a Nef- and ADAM17-Dependent Mechanism
Arenaccio, C., Chiozzini, C., Columba-Cabezas, S., Manfredi, F., Affabris, E., Baur, A. and Federico, M.
J. Virol., ***88(19)***, *11529-11539 (2014)*

Resting $CD4^+$ T lymphocytes resist human immunodeficiency virus (HIV) infection. Here, we provide evidence that exosomes from HIV-1-infected cells render resting human primary $CD4^+$ T lymphocytes permissive to HIV-1 replication. These results were obtained with transwell cocultures of HIV-1-infected cells with quiescent $CD4^+$ T lymphocytes in the presence of inhibitors of exosome release and were confirmed using exosomes purified from supernatants of HIV-1-infected primary $CD4^+$ T lymphocytes. We found that the expression of HIV-1 Nef in exosome-producing cells is both necessary and sufficient for cell activation as well as HIV-1 replication in target $CD4^+$ T lymphocytes. We also identified a Nef domain important for the effects we observed, i.e., the ^{62}EEEE^{65} acidic cluster domain. In addition, we observed that ADAM17, i.e., a disintegrin and metalloprotease converting pro-tumor necrosis factor alpha (TNF-α) in its mature form, associates with exosomes from HIV-1-infected cells, and plays a key role in the HIV-1 replication in quiescent $CD4^+$ T lymphocytes. Treatment with an inhibitor of ADAM17 abolished both activation and HIV-1 replication in resting $CD4^+$ T lymphocytes. TNF-α is the downstream effector of ADAM17 since the treatment of resting lymphocytes with anti-TNF-α antibodies blocked the HIV-1 replication. The data presented here are consistent with a model where Nef induces intercellular communication through exosomes to activate bystander quiescent $CD4^+$ T lymphocytes, thus stimulating viral spread.

3.2217 Diagnostic and Prognostic Potential of Extracellular Vesicles in Peripheral Blood
Revenfeld, A.L.S., Bæk, R., Hjuler Nielsen, M., Stensballe, A., Varming, K. and Jørgensen, M.
Clinical Therapeutics, ***36(6)***, *830-846 (2014)*

Purpose
Extracellular vesicles (EVs) are small, membrane-enclosed entities released from cells in many different biological systems. These vesicles play an important role in cellular communication by virtue of their protein, RNA, and lipid content, which can be transferred among cells. The complement of biomolecules reflects the parent cell, and their characterization may provide information about the presence of an aberrant process. Peripheral blood is a rich source of circulating EVs, which are easily accessible through a blood sample. An analysis of EVs in peripheral blood could provide access to unparalleled amounts of biomarkers of great diagnostic and prognostic value. The objectives of this review are to briefly present the current knowledge about EVs and to introduce a toolbox of selected techniques, which can be used to rapidly characterize clinically relevant properties of EVs from peripheral blood.
Methods
Several techniques exist to characterize the different features of EVs, including size, enumeration, RNA cargo, and protein phenotype. Each technique has a number of advantages and pitfalls. However, with the techniques presented in this review, a possible platform for EV characterization in a clinical setting is outlined.
Findings
Although EVs have great diagnostic and prognostic potential, a lack of standardization regarding EV analysis hampers the full use of this potential. Nevertheless, the analysis of EVs in peripheral blood has several advantages compared with traditional analyses of many soluble molecules in blood.
Implications
Overall, the use of EV analysis as a diagnostic and prognostic tool has prodigious clinical potential.

3.2218 Production of Outer Membrane Vesicles by the Plague Pathogen Yersinia pestis
Eddy, J.L., Gielda, L.M., Caulfield, A.J., Rangel, S.M. and Lathem, W.W.
PloS One, ***9(9)***, *e107002 (2014)*

Many Gram-negative bacteria produce outer membrane vesicles (OMVs) during cell growth and division, and some bacterial pathogens deliver virulence factors to the host via the release of OMVs during infection. Here we show that *Yersinia pestis*, the causative agent of the disease plague, produces and releases native OMVs under physiological conditions. These OMVs, approximately 100 nm in diameter, contain multiple virulence-associated outer membrane proteins including the adhesin Ail, the F1 outer fimbrial antigen, and the protease Pla. We found that OMVs released by *Y. pestis* contain catalytically active Pla that is competent for plasminogen activation and α2-antiplasmin degradation. The abundance of OMV-associated proteins released by *Y. pestis* is significantly elevated at 37°C compared to 26°C and is increased in response to membrane stress and mutations in RseA, Hfq, and the major Braun lipoprotein (Lpp). In addition, we show that *Y. pestis* OMVs are able to bind to components of the extracellular matrix such as fibronectin and laminin. These data suggest that *Y. pestis* may produce OMVs during mammalian infection and we propose that dispersal of Pla via OMV release may influence the outcome of infection through interactions with Pla substrates such as plasminogen and Fas ligand.

3.2219 Identification and Characterization of Outer Membrane Vesicle-Associated Proteins in Salmonella enterica Serovar Typhimurium
Bai, J., Kim, S.I., Ryu, S. and Yoon, H.
*Infect. Immun., **82(10)**, 4001-4010 (2014)*

Salmonella enterica serovar Typhimurium is a primary cause of enteric diseases and has acquired a variety of virulence factors during its evolution into a pathogen. Secreted virulence factors interact with commensal flora and host cells and enable Salmonella to survive and thrive in hostile environments. Outer membrane vesicles (OMVs) released from many Gram-negative bacteria function as a mechanism for the secretion of complex mixtures, including virulence factors. We performed a proteomic analysis of OMVs that were isolated under standard laboratory and acidic minimal medium conditions and identified 14 OMV-associated proteins that were observed in the OMV fraction isolated only under the acidic minimal medium conditions, which reproduced the nutrient-deficient intracellular milieu. The inferred roles of these 14 proteins were diverse, including transporter, enzyme, and transcriptional regulator. The absence of these proteins influenced Salmonella survival inside murine macrophages. Eleven of these proteins were predicted to possess secretion signal sequences at their N termini, and three (HupA, GlnH, and PhoN) of the proteins were found to be translocated into the cytoplasm of host cells. The comparative proteomic profiling of OMVs performed in this study revealed different protein compositions in the OMVs isolated under the two different conditions, which indicates that the OMV cargo depends on the growth conditions and provides a deeper insight into how Salmonella utilizes OMVs to adapt to environmental changes.

3.2220 Retroviral Retention Activates a Syk-Dependent HemITAM in Human Tetherin
Galao, R.P., Pickering, S., Curnock, R. and Neil, S.J.D.
*Cell Host & Microbe, **16**, 291-303 (2014)*

Tetherin (BST2/CD317) restricts the release of enveloped viral particles from infected cells. Coupled to this virion retention, hominid tetherins induce proinflammatory gene expression via activating NF-κB. We investigated the events initiating this tetherin-induced signaling and show that physical retention of retroviral particles induces the phosphorylation of conserved tyrosine residues in the cytoplasmic tails of tetherin dimers. This phosphorylation induces the recruitment of spleen tyrosine kinase (Syk), which is required for downstream NF-κB activation, indicating that the tetherin cytoplasmic tail resembles the hemi-immunoreceptor tyrosine-based activation motifs (hemITAMs) found in C-type lectin pattern recognition receptors. Retroviral-induced tetherin signaling is coupled to the cortical actin cytoskeleton via the Rac-GAP-containing protein RICH2 (ARHGAP44), and a naturally occurring tetherin polymorphism with reduced RICH2 binding exhibits decreased phosphorylation and NF-κB activation. Thus, upon virion retention, this linkage to the actin cytoskeleton likely triggers tetherin phosphorylation and subsequent signal transduction to induce an antiviral state.

3.2221 Epoxide-Mediated Differential Packaging of Cif and Other Virulence Factors into Outer Membrane Vesicles
Ballok, A.E., Filkins, L.M., Bomberger, J.M., Staton, B.A. and O'Toole, G.A.
*J. Bacteriol., **196(20)**, 3633-3642 (2014)*

Pseudomonas aeruginosa produces outer membrane vesicles (OMVs) that contain a number of secreted bacterial proteins, including phospholipases, alkaline phosphatase, and the CFTR inhibitory factor (Cif).

Previously, Cif, an epoxide hydrolase, was shown to be regulated at the transcriptional level by epoxides, which serve as ligands of the repressor, CifR. Here, we tested whether epoxides have an effect on Cif levels in OMVs. We showed that growth of P. aeruginosa in the presence of specific epoxides but not a hydrolysis product increased Cif packaging into OMVs in a CifR-independent fashion. The outer membrane protein, OprF, was also increased under these conditions, but alkaline phosphatase activity was not significantly altered. Additionally, we demonstrated that OMV shape and density were affected by epoxide treatment, with two distinct vesicle fractions present when cells were treated with epibromohydrin (EBH), a model epoxide. Vesicles isolated from the two density fractions exhibited different protein profiles in Western blotting and silver staining. We have shown that a variety of clinically or host-relevant treatments, including antibiotics, also alter the proteins packaged in OMVs. Proteomic analysis of purified OMVs followed by an analysis of transposon mutant OMVs yielded mutants with altered vesicle packaging. Finally, epithelial cell cytotoxicity was reduced in the vesicles formed in the presence of EBH, suggesting that this epoxide alters the function of the OMVs. Our data support a model whereby clinically or host-relevant signals mediate differential packaging of virulence factors in OMVs, which results in functional consequences for host-pathogen interactions.

3.2222 Leishmania donovani activates SREBP2 to modulate macrophage membrane cholesterol and mitochondrial oxidants for establishment of infection
Mukherjee, M., Ball, W.B. and Das, P.K.
*Int. J. Biochem. Cell Biol., **55**, 196-208 (2014)*

Establishment of infection by an intracellular pathogen depends on successful internalization with a concomitant neutralization of host defense machinery. *Leishmania donovani*, an intramacrophage pathogen, targets host SREBP2, a critical transcription factor, to regulate macrophage plasma membrane cholesterol and mitochondrial reactive oxygen species generation, favoring parasite invasion and persistence. *Leishmania* infection triggered membrane-raft reorientation-dependent Lyn-PI3K/Akt pathway activation which in turn deactivated GSK3 β to stabilize nuclear SREBP2. Moreover, cells perceiving less available intracellular cholesterol due to its sequestration at the plasma membrane resulted in the deregulation of the ER-residing SCAP-SREBP2-Insig circuit thereby assisting increased nuclear translocation of SREBP2. Both increased nuclear transport and stabilization of SREBP2 caused HMGCR-catalyzed cholesterol biosynthesis-mediated plasma membrane cholesterol enrichment leading to decreased membrane-fluidity and plausibly assisting delay in phagosomal acidification. Parasite survival ensuing entry was further ensured by SREBP2-dependent trasnscriptional up-regulation of UCP2, which suppressed mitochondrial ROS generation, one of the primary microbicidal molecules in macrophages recognized for its efficacy against *Leishmania*. Functional knock-down of SREBP2 both *in vitro* and *in vivo* was associated with reduction in macrophage plasma membrane cholesterol, increased ROS production and lower parasite survival. To our knowledge, this study, for the first time, reveals that *Leishmania* exploits macrophage cholesterol-dependent SREBP2 circuit to facilitate its entry and survival within the host.

3.2223 A Rab10:RalA G protein cascade regulates insulin-stimulated glucose uptake in adipocytes
Karunanithi, S., iong, T., Uhm, M., Leto, D., Sun, J., Chen, X-W. and Saltiel, A.R.
*Mol. Biol. Cell, **25**, 3059-3069 (2014)*

Insulin-stimulated glucose uptake in fat and muscle is mediated by the major facilitative glucose transporter Glut4. Insulin controls the trafficking of Glut4 to the plasma membrane via regulation of a series of small G proteins, including RalA and Rab10. We demonstrate here that Rab10 is a bona fide target of the GTPase-activating protein AS160, which is inhibited after phosphorylation by the protein kinase Akt. Once activated, Rab10 can increase the GTP binding of RalA by recruiting the Ral guanyl nucleotide exchange factor, Rlf/Rgl2. Rab10 and RalA reside in the same pool of Glut4-storage vesicles in untreated cells, and, together with Rlf, they ensure maximal glucose transport. Overexpression of membrane-tethered Rlf compensates for the loss of Rab10 in Glut4 translocation, suggesting that Rab10 recruits Rlf to membrane compartments for RalA activation and that RalA is downstream of Rab10. Together these studies identify a new G protein cascade in the regulation of insulin-stimulated Glut4 trafficking and glucose uptake.

3.2224 **Isolation and Functional Assessment of Eosinophil Crystalloid Granules**
Baptista-dos-Reis, R., Muniz, V.S. and Neves, J.S.
Methods in Mol. Biol., 1178, 93-100 (2014)

Cell-free granules, upon extrusion from human eosinophils, remain fully competent to secrete granule-derived proteins in receptor-mediated processes in response to different stimuli. However, in order to avoid the shrinkage and damage of granules, as well as preserve their structure, properties, and functionality, the use of an optimized process of subcellular fractionation using an isoosmotic density gradient is needed. Here, we describe a detailed protocol of subcellular fractionation of nitrogen-cavitated eosinophils on an isoosmotic iodinated density gradient (iodixanol) and the isolation of well-preserved and functional membrane-bound specific granules.

3.2225 **Role of LARP6 and Nonmuscle Myosin in Partitioning of Collagen mRNAs to the ER Membrane**
Wang, H. and Stefanovic, B.
*PloS One, **9(10)**, e108870 (2014)*

Type I collagen is extracellular matrix protein composed of two α1(I) and one α2(I) polypeptides that fold into triple helix. Collagen polypeptides are translated in coordination to synchronize the rate of triple helix folding to the rate of posttranslational modifications of individual polypeptides. This is especially important in conditions of high collagen production, like fibrosis. It has been assumed that collagen mRNAs are targeted to the membrane of the endoplasmic reticulum (ER) after translation of the signal peptide and by signal peptide recognition particle (SRP). Here we show that collagen mRNAs associate with the ER membrane even when translation is inhibited. Knock down of LARP6, an RNA binding protein which binds 5′ stem-loop of collagen mRNAs, releases a small amount of collagen mRNAs from the membrane. Depolimerization of nonmuscle myosin filaments has a similar, but stronger effect. In the absence of LARP6 or nonmuscle myosin filaments collagen polypeptides become hypermodified, are poorly secreted and accumulate in the cytosol. This indicates lack of coordination of their synthesis and retro-translocation due to hypermodifications and misfolding. Depolimerization of nonmuscle myosin does not alter the secretory pathway through ER and Golgi, suggesting that the role of nonmuscle myosin is primarily to partition collagen mRNAs to the ER membrane. We postulate that collagen mRNAs directly partition to the ER membrane prior to synthesis of the signal peptide and that LARP6 and nonmuscle myosin filaments mediate this process. This allows coordinated initiation of translation on the membrane bound collagen α1(I) and α2(I) mRNAs, a necessary step for proper synthesis of type I collagen.

3.2226 **The Get1/2 transmembrane complex is an endoplasmic-reticulum membrane protein insertase**
Wang, F., Chan, C., Weir, N.R. and Denic, V.
*Nature Letter, **512**, 441-444 (2014)*

Hundreds of tail-anchored proteins, including soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNAREs) involved in vesicle fusion, are inserted post-translationally into the endoplasmic reticulum membrane by a dedicated protein-targeting pathway[1, 2, 3, 4]. Before insertion, the carboxy-terminal transmembrane domains of tail-anchored proteins are shielded in the cytosol by the conserved targeting factor Get3 (in yeast; TRC40 in mammals)[5, 6, 7]. The Get3 endoplasmic-reticulum receptor comprises the cytosolic domains of the Get1/2 (WRB/CAML) transmembrane complex, which interact individually with the targeting factor to drive a conformational change that enables substrate release and, as a consequence, insertion[8, 9, 10, 11]. Because tail-anchored protein insertion is not associated with significant translocation of hydrophilic protein sequences across the membrane, it remains possible that Get1/2 cytosolic domains are sufficient to place Get3 in proximity with the endoplasmic-reticulum lipid bilayer and permit spontaneous insertion to occur[12, 13]. Here we use cell reporters and biochemical reconstitution to define mutations in the Get1/2 transmembrane domain that disrupt tail-anchored protein insertion without interfering with Get1/2 cytosolic domain function. These mutations reveal a novel Get1/2 insertase function, in the absence of which substrates stay bound to Get3 despite their proximity to the lipid bilayer; as a consequence, the notion of spontaneous transmembrane domain insertion is a non sequitur. Instead, the Get1/2 transmembrane domain helps to release substrates from Get3 by capturing their transmembrane domains, and these transmembrane interactions define a bona fide pre-integrated intermediate along a facilitated route for tail-anchor entry into the lipid bilayer. Our work sheds light on the fundamental point of convergence between co-translational and post-translational endoplasmic-reticulum membrane protein targeting and insertion: a mechanism for reducing the ability of a targeting factor to shield its substrates enables substrate handover to a transmembrane-domain-docking site embedded in the endoplasmic-reticulum membrane.

3.2227 **Membrane vesicle formation is associated with pyocin production under denitrifying conditions in Pseudomonas aeruginosa PAO1**
Toyofuku, M., Zhou, S., Sawada, I., Takaya, N., Uchiyama, H. and Nomura, N.
Environmental Microbiol., ***16(9)***, *2927-2938 (2014)*

Many Gram-negative bacteria produce membrane vesicles (MVs) that serve as vehicles to mediate intraspecies and interspecies interactions. Despite their ubiquity in Gram-negative bacteria and their biological importance, how MV formation is regulated is poorly understood. *Pseudomonas aeruginosa* is a ubiquitous bacterium that is one of the most extensively studied model organism in MVs. Recent studies highlight the importance of a quorum-sensing signal, *Pseudomonas* quinolone signal (PQS), in the formation of MVs; however, PQS synthesis requires oxygen and is not produced under anoxic conditions. This situation leads to the question of MV production under anoxic conditions. Here, we examined whether MVs are produced under denitrifying conditions and what kind of factors are involved in the MV production under such condition. Under denitrifying condition, *P. aeruginosa* PAO1 produced a considerable amount of MVs. Interestingly, pyocin components were found to be accumulated in the isolated MVs. Pyocin-related protein mutants produced less MVs compared with the wild type. We further indicate that pyocin production is activated by nitric oxide, in which the SOS response is involved. This study presents a regulatory mechanism where pyocin is associated with MV production, and further implies how the environment impacts MV production in *P. aeruginosa.*

3.2228 **Concentration of Sec12 at ER exit sites via interaction with cTAGE5 is required for collagen export**
Saito, K., Yamashiro, N., Tanabe, T., Kontani, K. and katada, T.
J. Cell Biol., ***206(6)***, *751-762 (2014)*

Mechanisms for exporting variably sized cargo from the endoplasmic reticulum (ER) using the same machinery remain poorly understood. COPII-coated vesicles, which transport secretory proteins from the ER to the Golgi apparatus, are typically 60–90 nm in diameter. However, collagen, which forms a trimeric structure that is too large to be accommodated by conventional transport vesicles, is also known to be secreted via a COPII-dependent process. In this paper, we show that Sec12, a guanine-nucleotide exchange factor for Sar1 guanosine triphosphatase, is concentrated at ER exit sites and that this concentration of Sec12 is specifically required for the secretion of collagen VII but not other proteins. Furthermore, Sec12 recruitment to ER exit sites is organized by its direct interaction with cTAGE5, a previously characterized collagen cargo receptor component, which functions together with TANGO1 at ER exit sites. These findings suggest that the export of large cargo requires high levels of guanosine triphosphate–bound Sar1 generated by Sec12 localized at ER exit sites.

3.2229 **The Atypical Cadherin Fat Directly Regulates Mitochondrial Function and Metabolic State**
Ssing, A., Tsatskis, Y., Fabian, L., Hester, I., Rosenfeld, R., Serricchio, M., Yau, N., Bietenhader, M., Shanbhag, R., Jurisicova, A., Brill, J.A., McQuibban, G.A. and McNeill, H.
Cell, ***158(6)***, *1293-1308 (2014)*

Fat (Ft) cadherins are enormous cell adhesion molecules that function at the cell surface to regulate the tumor-suppressive Hippo signaling pathway and planar cell polarity (PCP) tissue organization. Mutations in Ft cadherins are found in a variety of tumors, and it is presumed that this is due to defects in either Hippo signaling or PCP. Here, we show *Drosophila* Ft functions in mitochondria to directly regulate mitochondrial electron transport chain integrity and promote oxidative phosphorylation. Proteolytic cleavage releases a soluble 68 kDa fragment (Ft^{mito}) that is imported into mitochondria. Ft^{mito} binds directly to NADH dehydrogenase ubiquinone flavoprotein 2 (Ndufv2), a core component of complex I, stabilizing the holoenzyme. Loss of Ft leads to loss of complex I activity, increases in reactive oxygen species, and a switch to aerobic glycolysis. Defects in mitochondrial activity in *ft* mutants are independent of Hippo and PCP signaling and are reminiscent of the Warburg effect.

3.2230 **Clathrin Assembly Protein CALM Plays a Critical Role in KIT Signaling by Regulating Its Cellular Transport from Early to Late Endosomes in Hematopoietic Cells**
Rai, S., Tanaka, H., Suzuki, M., Ogoh, H., Taniguchi, Y., Morita, Y., Shimada, T., Tanimura, A., Matsui, K., Yokota, T., Oritani, K., Tanabe, K., Watanabe, T., Kanakura, Y. and Matsumura, I.
PloS One, ***9(10)***, *e109441 (2014)*

CALM is implicated in the formation of clathrin-coated vesicles, which mediate endocytosis and intracellular trafficking of growth factor receptors and nutrients. We previously found that *CALM*-deficient mice suffer from severe anemia due to the impaired clathrin-mediated endocytosis of transferrin receptor in immature erythroblast. However, CALM has been supposed to regulate the growth and survival of hematopoietic stem/progenitor cells. So, in this study, we focused on the function of CALM in these cells. We here show that the number of $Linage^{-}Sca\text{-}1^{+}KIT^{+}$ (LSK) cells decreased in the fetal liver of $CALM^{-/-}$ mice. Also, colony forming activity was impaired in $CALM^{-/-}$ LSK cells. In addition, SCF, FLT3, and TPO-dependent growth was severely impaired in $CALM^{-/-}$ LSK cells, while they can normally proliferate in response to IL-3 and IL-6. We also examined the intracellular trafficking of KIT using $CALM^{-/-}$ murine embryonic fibroblasts (MEFs) engineered to express KIT. At first, we confirmed that endocytosis of SCF-bound KIT was not impaired in $CALM^{-/-}$ MEFs by the internalization assay. However, SCF-induced KIT trafficking from early to late endosome was severely impaired in $CALM^{-/-}$ MEFs. As a result, although intracellular KIT disappeared 30 min after SCF stimulation in wild-type (WT) MEFs, it was retained in $CALM^{-/-}$ MEFs. Furthermore, SCF-induced phosphorylation of cytosolic KIT was enhanced and prolonged in $CALM^{-/-}$ MEFs compared with that in WT MEFs, leading to the excessive activation of Akt. Similar hyperactivation of Akt was observed in $CALM^{-/-}$ KIT^{+} cells. These results indicate that CALM is essential for the intracellular trafficking of KIT and its normal functions. Also, our data demonstrate that KIT located in the early endosome can activate downstream molecules as a signaling endosome. Because KIT activation is involved in the pathogenesis of some malignancies, the manipulation of CALM function would be an attractive therapeutic strategy.

3.2231 Modulation of Hepatitis C Virus Genome Replication by Glycosphingolipids and Four-Phosphate Adaptor Protein 2

Khan, I., Katikaneni, D.S., han, Q., Sanchez-Felipe, L., Hanada, K., Ambrose, R.L., macKenzie, J.M. and Konan, K.V.
J. Virol., ***88(21)***, *12276-12295 (2014)*

Hepatitis C virus (HCV) assembles its replication complex on cytosolic membrane vesicles often clustered in a membranous web (MW). During infection, HCV NS5A protein activates PI4KIIIα enzyme, causing massive production and redistribution of phosphatidylinositol 4-phosphate (PI4P) lipid to the replication complex. However, the role of PI4P in the HCV life cycle is not well understood. We postulated that PI4P recruits host effectors to modulate HCV genome replication or virus particle production. To test this hypothesis, we generated cell lines for doxycycline-inducible expression of short hairpin RNAs (shRNAs) targeting the PI4P effector, four-phosphate adaptor protein 2 (FAPP2). FAPP2 depletion attenuated HCV infectivity and impeded HCV RNA synthesis. Indeed, FAPP2 has two functional lipid-binding domains specific for PI4P and glycosphingolipids. While expression of the PI4P-binding mutant protein was expected to inhibit HCV replication, a marked drop in replication efficiency was observed unexpectedly with the glycosphingolipid-binding mutant protein. These data suggest that both domains are crucial for the role of FAPP2 in HCV genome replication. We also found that HCV significantly increases the level of some glycosphingolipids, whereas adding these lipids to FAPP2-depleted cells partially rescued replication, further arguing for the importance of glycosphingolipids in HCV RNA synthesis. Interestingly, FAPP2 is redistributed to the replication complex (RC) characterized by HCV NS5A, NS4B, or double-stranded RNA (dsRNA) foci. Additionally, FAPP2 depletion disrupts the RC and alters the colocalization of HCV replicase proteins. Altogether, our study implies that HCV coopts FAPP2 for virus genome replication via PI4P binding and glycosphingolipid transport to the HCV RC.

3.2232 Stearoyl Coenzyme A Desaturase 1 Is Associated with Hepatitis C Virus Replication Complex and Regulates Viral Replication

Nguuyen, L.N., Lim, Y-S., Pham, L.V., Shin, H-Y., Kim, Y-S. and Hwang, S.B.
J. Virol., ***88(21)***, *12311-12325 (2014)*

The hepatitis C virus (HCV) life cycle is tightly regulated by lipid metabolism of host cells. In order to identify host factors involved in HCV propagation, we have recently screened a small interfering RNA (siRNA) library targeting host genes that control lipid metabolism and lipid droplet formation using cell culture-grown HCV (HCVcc)-infected cells. We selected and characterized the gene encoding stearoyl coenzyme A (CoA) desaturase 1 (SCD1). siRNA-mediated knockdown or pharmacological inhibition of SCD1 abrogated HCV replication in both subgenomic replicon and Jc1-infected cells, while exogenous supplementation of either oleate or palmitoleate, products of SCD1 activity, resurrected HCV replication in SCD1 knockdown cells. SCD1 was coimmunoprecipitated with HCV nonstructural proteins and

colocalized with both double-stranded RNA (dsRNA) and HCV nonstructural proteins, indicating that SCD1 is associated with HCV replication complex. Moreover, SCD1 was fractionated and enriched with HCV nonstructural proteins at detergent-resistant membrane. Electron microscopy data showed that SCD1 is required for NS4B-mediated intracellular membrane rearrangement. These data further support the idea that SCD1 is associated with HCV replication complex and that its products may contribute to the proper formation and maintenance of membranous web structures in HCV replication complex. Collectively, these data suggest that manipulation of SCD1 activity may represent a novel host-targeted antiviral strategy for the treatment of HCV infection.

3.2233 **Proteome investigation of an organellar fraction of Toxoplasma gondii: a preliminary study**
Ferreira, D.da S., resende, I.T.M. and Lopez, J.A.
*BMC Proceedings, **8**(Suppl 4), P74 (2014)*

Toxoplasma gondii is a ubiquitous Apicomplexan parasite responsible for systemic diseases in both humans and animals. Toxoplasmosis is a major public health problem, infecting one-third of the world's human population and leading to abortion in domestic animals [1]. The search for new chemotherapeutic targets is imperative, due to its increasing resistance to the drugs currently available for combating this parasite [2]. Recent high-throughput
Proteomic approaches have provided a wealth of protein expression data on Apicomplexan parasites (e.g., T. gondii, Plasmodium falciparum), while a number of smaller-scale studies have examined specific drug-related hypotheses. Proteomic methods can be applied to study sub-cellular localization, cell function, organelle composition, changes in protein expression patterns in response to drug exposure, drug-protein binding, and validation of data from genomic annotation and transcript expression studies [3]. Organellar structures have therefore become potential targets for the parasite life cycle to control the levels of nutrients or salts that surround them [4]. The aim of this study was to perform a proteomic analysis of an organellar fraction of this Apicomplexan protozoan based on the structural and metabolic aspects.

3.2234 **ABCA1, ABCG1, and ABCG4 Are Distributed to Distinct Membrane Meso-Domains and Disturb Detergent-Resistant Domains on the Plasma Membrane**
Sano, O., Ito, S., kato, R., Shimizu, Y., Kobayashi, A., Kimura, Y., Kioka, N., hanada, K., Ueda, K. and Matsuo, M.
*PloS One, **9(10)**, e109886 (2014)*

ATP-binding cassette A1 (ABCA1), ABCG1, and ABCG4 are lipid transporters that mediate the efflux of cholesterol from cells. To analyze the characteristics of these lipid transporters, we examined and compared their distributions and lipid efflux activity on the plasma membrane. The efflux of cholesterol mediated by ABCA1 and ABCG1, but not ABCG4, was affected by a reduction of cellular sphingomyelin levels. Detergent solubility and gradient density ultracentrifugation assays indicated that ABCA1, ABCG1, and ABCG4 were distributed to domains that were solubilized by Triton X-100 and Brij 96, resistant to Triton X-100 and Brij 96, and solubilized by Triton X-100 but resistant to Brij 96, respectively. Furthermore, ABCG1, but not ABCG4, was colocalized with flotillin-1 on the plasma membrane. The amounts of cholesterol extracted by methyl-β-cyclodextrin were increased by ABCA1, ABCG1, or ABCG4, suggesting that cholesterol in non-raft domains was increased. Furthermore, ABCG1 and ABCG4 disturbed the localization of caveolin-1 to the detergent-resistant domains and the binding of cholera toxin subunit B to the plasma membrane. These results suggest that ABCA1, ABCG1, and ABCG4 are localized to distinct membrane meso-domains and disturb the meso-domain structures by reorganizing lipids on the plasma membrane; collectively, these observations may explain the different substrate profiles and lipid efflux roles of these transporters.

3.2235 **Endocytosis of Secreted Carboxyl Ester Lipase in a Syndrome of Diabetes and Pancreatic Exocrine Dysfunction**
Torsvik, J., Johansson, B.B., Dalva, M., Marie, M., Fjeld, K., Johansson, S., Bjørkøy, G., Saraste, J., Njølstad, P.R. and Molven, A.
*J. Biol. Chem., **289**, 29097-29111 (2014)*

Maturity-onset diabetes of the young, type 8 (MODY8) is characterized by a syndrome of autosomal dominantly inherited diabetes and exocrine pancreatic dysfunction. It is caused by deletion mutations in the last exon of the carboxyl ester lipase (*CEL*) gene, resulting in a CEL protein with increased tendency to aggregate. In this study we investigated the intracellular distribution of the wild type (WT) and mutant (MUT) CEL proteins in cellular models. We found that both CEL-WT and CEL-MUT were secreted via

the endoplasmic reticulum and Golgi compartments. However, their subcellular distributions differed, as only CEL-MUT was observed as an aggregate at the cell surface and inside large cytoplasmic vacuoles. Many of the vacuoles were identified as components of the endosomal system, and after its secretion, the mutant CEL protein was re-internalized, transported to the lysosomes, and degraded. Internalization of CEL-MUT also led to reduced viability of pancreatic acinar and beta cells. These findings may have implications for the understanding of how the acinar-specific CEL-MUT protein causes both exocrine and endocrine pancreatic disease.

3.2236 Hallmarks of Hepatitis C Virus in Equine Hepacivirus

Tanaka, T., Kasai, H., Yamashita, A., Okuyama-Dobashi, K., Yasumoto, J., maekawa, S., Enomoto, N., Okamoto, T., Matsuura, Y., Morimatsu, M., Manabe, N., Ochiai, K., Yamashita, K. and Moriishi, K.
J. Virol., ***88(22)***, *13352-13366 (2014)*

Equine hepacivirus (EHcV) has been identified as a closely related homologue of hepatitis C virus (HCV) in the United States, the United Kingdom, and Germany, but not in Asian countries. In this study, we genetically and serologically screened 31 serum samples obtained from Japanese-born domestic horses for EHcV infection and subsequently identified 11 PCR-positive and 7 seropositive serum samples. We determined the full sequence of the EHcV genome, including the 3′ untranslated region (UTR), which had previously not been completely revealed. The polyprotein of a Japanese EHcV strain showed approximately 95% homology to those of the reported strains. HCV-like *cis*-acting RNA elements, including the stem-loop structures of the 3′ UTR and kissing-loop interaction were deduced from regions around both UTRs of the EHcV genome. A comparison of the EHcV and HCV core proteins revealed that Ile^{190} and Phe^{191} of the EHcV core protein could be important for cleavage of the core protein by signal peptide peptidase (SPP) and were replaced with Ala and Leu, respectively, which inhibited intramembrane cleavage of the EHcV core protein. The loss-of-function mutant of SPP abrogated intramembrane cleavage of the EHcV core protein and bound EHcV core protein, suggesting that the EHcV core protein may be cleaved by SPP to become a mature form. The wild-type EHcV core protein, but not the SPP-resistant mutant, was localized on lipid droplets and partially on the lipid raft-like membrane in a manner similar to that of the HCV core protein. These results suggest that EHcV may conserve the genetic and biological properties of HCV.

3.2237 Nuclear Enrichment of Folate Cofactors and Methylenetetrahydrofolate Dehydrogenase 1 (MTHFD1) Protect de Novo Thymidylate Biosynthesis during Folate Deficiency

Field, M.S., Kamynina, E., Agunloye, O.C., Liebenthal, R.P., lamarre, S.G., Brosnan, M.E., Brosnan, J.T. and Stover, P.J.
J. Biol. Chem., ***289(43)***, *29642-29650 (2014)*

Folate-mediated one-carbon metabolism is a metabolic network of interconnected pathways that is required for the *de novo* synthesis of three of the four DNA bases and the remethylation of homocysteine to methionine. Previous studies have indicated that the thymidylate synthesis and homocysteine remethylation pathways compete for a limiting pool of methylenetetrahydrofolate cofactors and that thymidylate biosynthesis is preserved in folate deficiency at the expense of homocysteine remethylation, but the mechanisms are unknown. Recently, it was shown that thymidylate synthesis occurs in the nucleus, whereas homocysteine remethylation occurs in the cytosol. In this study we demonstrate that methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), an enzyme that generates methylenetetrahydrofolate from formate, ATP, and NADPH, functions in the nucleus to support *de novo* thymidylate biosynthesis. MTHFD1 translocates to the nucleus in S-phase MCF-7 and HeLa cells. During folate deficiency mouse liver MTHFD1 levels are enriched in the nucleus >2-fold at the expense of levels in the cytosol. Furthermore, nuclear folate levels are resistant to folate depletion when total cellular folate levels are reduced by >50% in mouse liver. The enrichment of folate cofactors and MTHFD1 protein in the nucleus during folate deficiency in mouse liver and human cell lines accounts for previous metabolic studies that indicated 5,10-methylenetetrahydrofolate is preferentially directed toward *de novo* thymidylate biosynthesis at the expense of homocysteine remethylation during folate deficiency.

3.2238 β-Elemene against human lung cancer via up-regulation of P53 protein expression to promote the release of exosome

Li, J., Yu, J. and wang, Y.
Lung Cancer, ***86***, *144-150 (2014)*

Background

β-Elemene, a novel antitumor plant drug extracted from the traditional Chinese medicinal herb Zedoary, has been shown to be effective against a wide variety of tumors. Recent studies have indicated that β-elemene can inhibit the growth of lung cancer cells; however, the exact mechanism of β-element's action in lung cancer remains largely unknown. In the present study, the antitumor effect of β-elemene on human lung cancer cells and the mechanism involved has been investigated.
Methods
The inhibitory effects of β-elemene on cell growth were measured by Trypan Blue exclusion and MTT assay. Flow cytometric analysis was used to detect the cells' apoptotic rate. The expression of P53 mRNA and protein were measured by RT-PCR and Western blot analysis, respectively. Exosomes were isolated by differential centrifugation steps and analyzed by electron microscopy and western blotting. P53 knockdown cells were established through transfection with P53 siRNA. To investigate the effect of β-elemene on the tumor growth *in vivo*, a Xenograft nude mouse model was established by injecting the A549 cells into the back of a BABL/c nude mouse.
Results
β-Elemene markedly inhibited growth and induced apoptosis in lung cancer cells. The levels of the anti-apoptotic genes Bcl-2 and Bcl-xl in A549 cells decreased, while expression of P53 and production of exosomes increased after β-elemene treatment. Further siRNA studies suggested that the effect of β-elemene on A549 cells is dependent on P53 expression. Exosomes derived from A549 cultured with a human lung cancer cell line exhibited decreased tumor cell proliferation. The *in vivo* study demonstrated that β-elemene inhibited tumor growth, and up-regulated the expression of P53 and the release of exosome.
Conclusion
Our results demonstrated β-elemene acts on lung cancer cells in a P53 dependent manner and exosomes are involved in the regulation of cell proliferation.

3.2239 Apolipoprotein J, a glucose-upregulated molecular chaperone, stabilizes core and NS5A to promote infectious hepatitis C virus virion production
Lin, C-C., Tsai, P., Sun, H-Y., Hsu, M-C., Lee, J-C., Wu, I-C., Tsao, C-W., Chang, T-T. and Young, K-C.
J. Hepatol., ***61***, *984-993 (2014)*

Background & Aims
Hepatitis C virus (HCV) infection leads to glucose abnormality. HCV depends on lipid droplets (LDs) and very-low density lipoproteins for assembly/releasing; however, the components and locations for this process remain unidentified. Apolipoprotein J (ApoJ), upregulated by glucose, functions as Golgi chaperone of secreted proteins and resides abundantly in very-low density lipoproteins. This study investigates the interplay between glucose, ApoJ and HCV virion production.
Methods
The effects of high glucose on ApoJ expression and HCV production were evaluated with cultivated HuH7.5, primary human hepatocytes, and in treatment naive chronic hepatitis C patients. How ApoJ affects HCV lifecycle was assessed using siRNA knockdown strategy in JFH1 infected and subgenomic replicon cells. The interactions and locations of ApoJ with viral and host components were examined by immunoprecipitation, immunofluorescence and subcellular fractionation experiments.
Results
HCV infection increased ApoJ expression, which in parallel with HCV infectivity was additionally elevated with high glucose treatment. Serum ApoJ correlated positively with fasting blood glucose concentration and HCV-RNA titre in patients. ApoJ silencing reduced intracellular and extracellular HCV infectivity and extracellular HCV-RNA, but accumulated intracellular HCV-RNA in HCV-infected cells. ApoJ interacted with HCV core and NS5A and stabilized the dual protein complex. HCV infection dispersed cytoplasmic ApoJ from the compact zones of the Golgi to encircle LDs, where co-localization of the core, NS5A, HCV-RNA, subcellular markers for LDs, endoplasmic reticulum (ER), Golgi, and membrane contact sites occurred.
Conclusions
ApoJ facilitates infectious HCV particle production via stabilization of core/NS5A, which might surround LDs at the ER-Golgi membrane contact site.

3.2240 HTLV-1 Tax Stabilizes MCL-1 via TRAF6-Dependent K63-Linked Polyubiquitination to Promote Cell Survival and Transformation
Choi, Y.B. and Harhaj, E.W.

*PloS Pathogens, **10(10)**, e1004458 (2014)*

The human T-cell leukemia virus type 1 (HTLV-1) Tax protein hijacks the host ubiquitin machinery to activate IκB kinases (IKKs) and NF-κB and promote cell survival; however, the key ubiquitinated factors downstream of Tax involved in cell transformation are unknown. Using mass spectrometry, we undertook an unbiased proteome-wide quantitative survey of cellular proteins modified by ubiquitin in the presence of Tax or a Tax mutant impaired in IKK activation. Tax induced the ubiquitination of 22 cellular proteins, including the anti-apoptotic BCL-2 family member MCL-1, in an IKK-dependent manner. Tax was found to promote the nondegradative lysine 63 (K63)-linked polyubiquitination of MCL-1 that was dependent on the E3 ubiquitin ligase TRAF6 and the IKK complex. Tax interacted with and activated TRAF6, and triggered its mitochondrial localization, where it conjugated four carboxyl-terminal lysine residues of MCL-1 with K63-linked polyubiquitin chains, which stabilized and protected MCL-1 from genotoxic stress-induced degradation. TRAF6 and MCL-1 played essential roles in the survival of HTLV-1 transformed cells and the immortalization of primary T cells by HTLV-1. Therefore, K63-linked polyubiquitination represents a novel regulatory mechanism controlling MCL-1 stability that has been usurped by a viral oncogene to precipitate cell survival and transformation.

3.2241 **Label-Free Protein Quantification for Plant Golgi Protein Localization and Abundance**
Nikolovski, N., Shliaha, P.V., Gatto, L., Dupree, P. and Lilley, K.S.
*Plant Physiol., **166**, 1033-1043 (2014)*

The proteomic composition of the Arabidopsis (*Arabidopsis thaliana*) Golgi apparatus is currently reasonably well documented; however, little is known about the relative abundances between different proteins within this compartment. Accurate quantitative information of Golgi resident proteins is of great importance: it facilitates a better understanding of the biochemical processes that take place within this organelle, especially those of different polysaccharide synthesis pathways. Golgi resident proteins are challenging to quantify because the abundance of this organelle is relatively low within the cell. In this study, an organelle fractionation approach targeting the Golgi apparatus was combined with a label-free quantitative mass spectrometry (data-independent acquisition method using ion mobility separation known as LC-IMS-MS^E [or $HDMS^E$]) to simultaneously localize proteins to the Golgi apparatus and assess their relative quantity. In total, 102 Golgi-localized proteins were quantified. These data show that organelle fractionation in conjunction with label-free quantitative mass spectrometry is a powerful and relatively simple tool to access protein organelle localization and their relative abundances. The findings presented open a unique view on the organization of the plant Golgi apparatus, leading toward unique hypotheses centered on the biochemical processes of this organelle.

3.2242 **Exosome Analysis: A Promising Biomarker System with Special Attention to Saliva**
Zheng, X., Chen, F., Zhang, J., Zhang, Q. and Lin, J.
*J. Membrane Biol., **247**, 1129-1136 (2014)*

Today, exosome-related studies have become a focus in science and technology. Recently, three scientists won the Nobel Prize for determining the mechanisms of exosomal transport, making exosomes a promising biomarker system for disease diagnosis and treatment. This review provides a general introduction of exosomes and explores the recent progress on the function, application, isolation, and identification of exosomes as biomarkers in blood and other body fluids, especially in saliva. Detailed information of exosomal proteins and RNAs is discussed in the paper because of their ability to determine the function of exosomes. Due to their noninvasive assessment for quick and convenient diagnosis of diseases, salivary exosomes may well be promising biomarkers.

3.2243 **Subcellular localization of coagulation factor II receptor-like 1 in neurons governs angiogenesis**
Joyal, J-S. et al
*Nature Med., **20(10)**, 1165-1173 (2014)*

Neurons have an important role in retinal vascular development. Here we show that the G protein–coupled receptor (GPCR) coagulation factor II receptor-like 1 (F2rl1, previously known as Par2) is abundant in retinal ganglion cells and is associated with new blood vessel formation during retinal development and in ischemic retinopathy. After stimulation, F2rl1 in retinal ganglion cells translocates from the plasma membrane to the cell nucleus using a microtubule-dependent shuttle that requires sorting nexin 11 (Snx11). At the nucleus, F2rl1 facilitates recruitment of the transcription factor Sp1 to trigger *Vegfa* expression and, in turn, neovascularization. In contrast, classical plasma membrane activation of F2rl1 leads to the

expression of distinct genes, including *Ang1*, that are involved in vessel maturation. Mutant versions of F2rl1 that prevent nuclear relocalization but not plasma membrane activation interfere with *Vegfa* but not *Ang1* expression. Complementary angiogenic factors are therefore regulated by the subcellular localization of a receptor (F2rl1) that governs angiogenesis. These findings may have implications for the selectivity of drug actions based on the subcellular distribution of their targets.

3.2244 KIF13B regulates angiogenesis through Golgi to plasma membrane trafficking of VEGFR2
Yamada, K.H., nakajima, Y., Geyer, M., Wary, K.K., Ushio-Fukai, M., Komarova, Y. and Malik, A.B.
J. Cell Sci., ***127(20)***, *4518-4530 (2014)*

Although the trafficking of newly synthesized VEGFR2 to the plasma membrane is a key determinant of angiogenesis, the molecular mechanisms of Golgi to plasma membrane trafficking are unknown. Here, we have identified a key role of the kinesin family plus-end molecular motor KIF13B in delivering VEGFR2 cargo from the Golgi to the endothelial cell surface. KIF13B is shown to interact directly with VEGFR2 on microtubules. We also observed that overexpression of truncated versions of KIF13B containing the binding domains that interact with VEGFR2 inhibited VEGF-induced capillary tube formation. KIF13B depletion prevented VEGF-mediated endothelial migration, capillary tube formation and neo-vascularization in mice. Impairment in trafficking induced by knockdown of *KIF13B* shunted VEGFR2 towards the lysosomal degradation pathway. Thus, KIF13B is an essential molecular motor required for the trafficking of VEGFR2 from the Golgi, and its delivery to the endothelial cell surface mediates angiogenesis.

3.2245 Lipid rafts couple class A scavenger receptors to phospholipase A2 activation during macrophage adhesion
Vadali, S. and Post, S.R.
J. Leukoc. Biol., ***96(5)***, *873-881 (2014)*

SR-A mediated macrophage adhesion to modified ECM proteins in a process that involves physical attachment of SR-A to modified ECM and activation of Lyn-PI3K and PLA_2-12/15-lipoxygenase signaling pathways. Structurally, SR-A-mediated cell adhesion requires a 6-aa membrane-proximal cytoplasmic motif. However, the mechanism that couples SR-A-mediated adhesion to activation of these distinct signaling pathways is not known. For other adhesion receptors, including integrins, localization in cholesterol-rich LRs is an important mechanism for coupling the receptor with the activation of specific signaling pathways. We hypothesized that SR-A-mediated macrophage adhesion might also involve LRs. Our results demonstrate that SR-A is enriched in LRs in HEK cells that heterologously express SR-A and in macrophages that endogenously expressed the receptor. We further show that a truncated SR-A construct ($SR\text{-}A_{\Delta 1-49}$), which mediates cell adhesion but not ligand internalization, is also enriched in LRs, suggesting an association between LRs and SR-A-dependent cell adhesion. To examine this association more directly, we used the cholesterol chelator MβCD to deplete cholesterol and disrupt LR function. We found that cholesterol depletion significantly decreased SR-A-mediated macrophage adhesion. We further show that decreased SR-A-dependent macrophage adhesion following cholesterol depletion results from the inhibition of PLA_2 but not PI3K activation. Overall, our results demonstrate an important role for LRs in selectively coupling SR-A with PLA_2 activation during macrophage adhesion.

3.2246 Plasma exosomal α-synuclein is likely CNS-derived and increased in Parkinson's disease
Shi, M. et al
Acta Neuropathol., ***128(5)***, *639-650 (2014)*

Extracellular α-synuclein is important in the pathogenesis of Parkinson's disease (PD) and also as a potential biomarker when tested in the cerebrospinal fluid (CSF). The performance of blood plasma or serum α-synuclein as a biomarker has been found to be inconsistent and generally ineffective, largely due to the contribution of peripherally derived α-synuclein. In this study, we discovered, via an intracerebroventricular injection of radiolabeled α-synuclein into mouse brain, that CSF α-synuclein was readily transported to blood, with a small portion being contained in exosomes that are relatively specific to the central nervous system (CNS). Consequently, we developed a technique to evaluate the levels of α-synuclein in these exosomes in individual plasma samples. When applied to a large cohort of clinical samples (267 PD, 215 controls), we found that in contrast to CSF α-synuclein concentrations, which are consistently reported to be lower in PD patients compared to controls, the levels of plasma exosomal α-synuclein were substantially higher in PD patients, suggesting an increased efflux of the protein to the peripheral blood of these patients. Furthermore, although no association was observed between plasma

exosomal and CSF α-synuclein, a significant correlation between plasma exosomal α-synuclein and disease severity (r = 0.176, p = 0.004) was observed, and the diagnostic sensitivity and specificity achieved by plasma exosomal α-synuclein were comparable to those determined by CSF α-synuclein. Further studies are clearly needed to elucidate the mechanism involved in the transport of CNS α-synuclein to the periphery, which may lead to a more convenient and robust assessment of PD clinically.

3.2247 CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling
Platt, R. et al
*Cell, **159(2)**, 440-455 (2014)*

CRISPR-Cas9 is a versatile genome editing technology for studying the functions of genetic elements. To broadly enable the application of Cas9 in vivo, we established a Cre-dependent Cas9 knockin mouse. We demonstrated in vivo as well as ex vivo genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells. Using these mice, we simultaneously modeled the dynamics of *KRAS*, *p53*, and *LKB1*, the top three significantly mutated genes in lung adenocarcinoma. Delivery of a single AAV vector in the lung generated loss-of-function mutations in *p53* and *Lkb1*, as well as homology-directed repair-mediated $Kras^{G12D}$ mutations, leading to macroscopic tumors of adenocarcinoma pathology. Together, these results suggest that Cas9 mice empower a wide range of biological and disease modeling applications.

3.2248 p130Cas Scaffolds the Signalosome To Direct Adaptor-Effector Cross Talk during Kaposi's Sarcoma-Associated Herpesvirus Trafficking in Human Microvascular Dermal Endothelial Cells
Bandyopadhyay, C., Veetil, M.V., Dutta, S. and Chandran, B.
*J. Virol., **88(23)**, 13858-13878 (2014)*

Kaposi's sarcoma-associated herpesvirus (KSHV) interacts with cell surface receptors, such as heparan sulfate, integrins (α3β1, αVβ3, and αVβ5), and EphrinA2 (EphA2), and activates focal adhesion kinase (FAK), Src, phosphoinositol 3-kinase (PI3-K), c-Cbl, and RhoA GTPase signal molecules early during lipid raft (LR)-dependent productive macropinocytic entry into human dermal microvascular endothelial cells. Our recent studies have identified CIB1 as a signal amplifier facilitating EphA2 phosphorylation and subsequent cytoskeletal cross talk during KSHV macropinocytosis. Although CIB1 lacks an enzymatic activity and traditional adaptor domain or known interacting sequence, it associated with the KSHV entry signal complex and the CIB1-KSHV association was sustained over 30 min postinfection. To identify factors scaffolding the EphA2-CIB1 signal axis, the role of major cellular scaffold protein p130Cas (Crk-associated substrate of Src) was investigated. Inhibitor and small interfering RNA (siRNA) studies demonstrated that KSHV induced p130Cas in an EphA2-, CIB1-, and Src-dependent manner. p130Cas and Crk were associated with KSHV, LRs, EphA2, and CIB1 early during infection. Live-cell microscopy and biochemical studies demonstrated that p130Cas knockdown did not affect KSHV entry but significantly reduced productive nuclear trafficking of viral DNA and routed KSHV to lysosomal degradation. p130Cas aided in scaffolding adaptor Crk to downstream guanine nucleotide exchange factor phospho-C3G possibly to coordinate GTPase signaling during KSHV trafficking. Collectively, these studies demonstrate that p130Cas acts as a bridging molecule between the KSHV-induced entry signal complex and the downstream trafficking signalosome in endothelial cells and suggest that simultaneous targeting of KSHV entry receptors with p130Cas would be an attractive potential avenue for therapeutic intervention in KSHV infection.

3.2249 Magnetic Nanoparticles to Recover Cellular Organelles and Study the Time Resolved Nanoparticle-Cell Interactome throughout Uptake
Bertoli, F., Davies, G-L., Monopoli, M.P., Moloney, M., Gun'ko, Y.K., Salvati, A. and Dawson, K.A.
*Small, **10(16)**, 3307-3315 (2014)*

Nanoparticles in contact with cells and living organisms generate quite novel interactions at the interface between the nanoparticle surface and the surrounding biological environment. However, a detailed time resolved molecular level description of the evolving interactions as nanoparticles are internalized and trafficked within the cellular environment is still missing and will certainly be required for the emerging arena of nanoparticle-cell interactions to mature. In this paper promising methodologies to map out the time resolved nanoparticle-cell interactome for nanoparticle uptake are discussed. Thus silica coated magnetite nanoparticles are presented to cells and their magnetic properties used to isolate, in a time resolved manner, the organelles containing the nanoparticles. Characterization of the recovered fractions shows that different cell compartments are isolated at different times, in agreement with imaging results on

nanoparticle intracellular location. Subsequently the internalized nanoparticles can be further isolated from the recovered organelles, allowing the study of the most tightly nanoparticle-bound biomolecules, analogous to the 'hard corona' that so far has mostly been characterized in extracellular environments. Preliminary data on the recovered nanoparticles suggest that significant portion of the original corona (derived from the serum in which particles are presented to the cells) is preserved as nanoparticles are trafficked through the cells.

3.2250 The Quantitative Nuclear Matrix Proteome as a Biochemical Snapshot of Nuclear Organization
Engelke, R., Riede, J., Hegermann, J., Wuerch, A., Eimer, S., Dengjel, J. and Mittler, G.
*J. Proteome, **13(9)**, 3940-3956 (2014)*

The nuclear matrix (NM) is an operationally defined structure of the mammalian cell nucleus that resists stringent biochemical extraction procedures applied subsequent to nuclease-mediated chromatin digestion of intact nuclei. This comprises removal of soluble biomolecules and chromatin by means of either detergent (LIS: lithium diiodosalicylate) or high salt (AS: ammonium sulfate, sodium chloride) treatment. So far, progress toward defining *bona fide* NM proteins has been hindered by the problem of distinguishing them from copurifying abundant contaminants and extraction-method-intrinsic precipitation artifacts. Here, we present a highly improved NM purification strategy, adding a FACS sorting step for efficient isolation of morphologically homogeneous lamin B positive NM specimens. SILAC-based quantitative proteome profiling of LIS-, AS-, or NaCl-extracted matrices versus the nuclear proteome together with rigorous statistical filtering enables the compilation of a high-quality catalogue of NM proteins commonly enriched among the three different extraction methods. We refer to this set of 272 proteins as the NM central proteome. Quantitative NM retention profiles for 2381 proteins highlight elementary features of nuclear organization and correlate well with immunofluorescence staining patterns reported in the Human Protein Atlas, demonstrating that the NM central proteome is significantly enriched in proteins exhibiting a nuclear body as well as nuclear speckle-like morphology.

3.2251 Hepatic fatty acid uptake is regulated by the sphingolipid acyl chain length
Park, W-J., park, J-W., Merrill Jr., A.H., Storchm J., Pewzner-Jung, Y. and Futerman, A.H.
*Biochim.Biophys. Acta, **1841**, 1754-1766 (2014)*

Ceramide synthase 2 (CerS2) null mice cannot synthesize very-long acyl chain (C22‑C24) ceramides resulting in significant alterations in the acyl chain composition of sphingolipids. We now demonstrate that hepatic triacylglycerol (TG) levels are reduced in the liver but not in the adipose tissue or skeletal muscle of the CerS2 null mouse, both before and after feeding with a high fat diet (HFD), where no weight gain was observed and large hepatic nodules appeared. Uptake of both BODIPY-palmitate and [^{3}H]-palmitate was also abrogated in the hepatocytes and liver. The role of a number of key proteins involved in fatty acid uptake was examined, including FATP5, CD36/FAT, FABPpm and cytoplasmic FABP1. Levels of FATP5 and FABP1 were decreased in the CerS2 null mouse liver, whereas CD36/FAT levels were significantly elevated and CD36/FAT was also mislocalized upon insulin treatment. Moreover, treatment of hepatocytes with C22‑C24-ceramides down-regulated CD36/FAT levels. Infection of CerS2 null mice with recombinant adeno-associated virus (rAAV)-CerS2 restored normal TG levels and corrected the mislocalization of CD36/FAT, but had no effect on the intracellular localization or levels of FATP5 or FABP1. Together, these results demonstrate that hepatic fatty acid uptake via CD36/FAT can be regulated by altering the acyl chain composition of sphingolipids.

3.2252 Role of the nucleocapsid region in HIV-1 Gag assembly as investigated by quantitative fluorescence-based microscopy
De Rocquigny, H., El Meshri, S.E., Richert, L., Didier, P., Darlix, J-L. and Mely, Y.
*Virus Res., **193**, 78-88 (2014)*

The Gag precursor of HIV-1, formed of the four proteic regions matrix (MA), capsid (CA), nucleocapsid (NC) and p6, orchestrates virus morphogenesis. This complex process relies on three major interactions, NC-RNA acting as a scaffold, CA-CA and MA-membrane that targets assembly to the plasma membrane (PM). The characterization of the molecular mechanism of retroviral assembly has extensively benefited from biochemical studies and more recently an important step forward was achieved with the use of fluorescence-based techniques and fluorescently labeled viral proteins. In this review, we summarize the findings obtained with such techniques, notably quantitative-based approaches, which highlight the role of the NC region in Gag assembly.

3.2253 Cystatin C protects neuronal cells against mutant copper-zinc superoxide dismutase-mediated toxicity

Watenabe, S., Hayakawa, ST., Wakasugi, K. and Yamanaka, K.
*Cell Death and Disease, **5**, e1497 (2014)*

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective and progressive loss of motor neurons. Cystatin C (CysC), an endogenous cysteine protease inhibitor, is a major protein component of Bunina bodies observed in the spinal motor neurons of sporadic ALS and is decreased in the cerebrospinal fluid of ALS patients. Despite prominent deposition of CysC in ALS, the roles of CysC in the central nervous system remain unknown. Here, we identified the neuroprotective activity of CysC against ALS-linked mutant Cu/Zn-superoxide dismutase (SOD1)-mediated toxicity. We found that exogenously added CysC protected neuronal cells including primary cultured motor neurons. Moreover, the neuroprotective property of CysC was dependent on the coordinated activation of two distinct pathways: autophagy induction through AMPK-mTOR pathway and inhibition of cathepsin B. Furthermore, exogenously added CysC was transduced into the cells and aggregated in the cytosol under oxidative stress conditions, implying a relationship between the neuroprotective activity of CysC and Bunina body formation. These data suggest CysC is an endogenous neuroprotective agent and targeting CysC in motor neurons may provide a novel therapeutic strategy for ALS.

3.2254 A Conserved Endoplasmic Reticulum Membrane Protein Complex (EMC) Facilitates Phospholipid Transfer from the ER to Mitochondria

Lahiri, S., Chao, J.T., Tavassoli, S., Wong, A.K.O., Choudhary, V., Young, B.P., Loewen, C.J.R. and Prinz, W.A.
*PloS Biology, **12(10)**, e1001969 (2014)*

Mitochondrial membrane biogenesis and lipid metabolism require phospholipid transfer from the endoplasmic reticulum (ER) to mitochondria. Transfer is thought to occur at regions of close contact of these organelles and to be nonvesicular, but the mechanism is not known. Here we used a novel genetic screen in *S. cerevisiae* to identify mutants with defects in lipid exchange between the ER and mitochondria. We show that a strain missing multiple components of the conserved ER membrane protein complex (EMC) has decreased phosphatidylserine (PS) transfer from the ER to mitochondria. Mitochondria from this strain have significantly reduced levels of PS and its derivative phosphatidylethanolamine (PE). Cells lacking EMC proteins and the ER–mitochondria tethering complex called ERMES (the ER–mitochondria encounter structure) are inviable, suggesting that the EMC also functions as a tether. These defects are corrected by expression of an engineered ER–mitochondrial tethering protein that artificially tethers the ER to mitochondria. EMC mutants have a significant reduction in the amount of ER tethered to mitochondria even though ERMES remained intact in these mutants, suggesting that the EMC performs an additional tethering function to ERMES. We find that all Emc proteins interact with the mitochondrial translocase of the outer membrane (TOM) complex protein Tom5 and this interaction is important for PS transfer and cell growth, suggesting that the EMC forms a tether by associating with the TOM complex. Together, our findings support that the EMC tethers ER to mitochondria, which is required for phospholipid synthesis and cell growth.

3.2255 In Candida albicans hyphae, Sec2p is physically associated with SEC2 mRNA on secretory vesicles

Caballero-Lima, D., Hautbergue, G.M., Wilson, S.A. and Sudbery, P.E.
*Mol. Microbiol., **94(4)**, 828-842 (2014)*

Candida albicans hyphae grow in a highly polarized fashion from their tips. This polarized growth requires the continuous delivery of secretory vesicles to the tip region. Vesicle delivery depends on Sec2p, the Guanine Exchange Factor (GEF) for the Rab GTPase Sec4p. GTP bound Sec4p is required for the transit of secretory vesicles from the trans-Golgi to sites of polarized growth. We previously showed that phosphorylation of Sec2p at residue S584 was necessary for Sec2p to support hyphal, but not yeast growth. Here we show that on secretory vesicles *SEC2* mRNA is physically associated with Sec2p. Moreover, we show that the phosphorylation of S584 allows *SEC2* mRNA to dissociate from Sec2p and we speculate that this is necessary for Sec2p function and/or translation. During hyphal extension, the growing tip may be separated from the nucleus by up to 15 μm. Transport of *SEC2* mRNA on secretory vesicles to the tip localizes *SEC2* translation to tip allowing a sufficient accumulation of this key protein at the site of polarized growth.

3.2256 290 Exosome analysis in cancer patients: From the preclinical towards the clinical application: Trial design

Mertens, I., Castiglia, M., Carreca, A.P., baggertman, G., Peeters, M., Pauwels, P. and Rolfo, C.
Eur. J. Cancer., ***50****, Suppl. 6, 96 (2014)*

Background: Cancer cells produce a heterogeneous mixture of vesicular, organelle-like structures (extracellular vesicles, EVs) into their surroundings including blood and other body fluids. Exosomes are small (40 to 100 nm) membrane derived vesicles that develop from exophytic budding of the cellular membrane after the fusion of multivesicular bodies or mature endosomes with the cellular membrane. It has been shown that tumour cells exposed to hypoxia secrete exosomes with enhanced angiogenic and metastatic potential. Thus exosomes might be involved in tumor progression and they can potentially be used for prognosis and therapy selection, as they contain a variety of molecules such as signal proteins and/or peptides, microRNAs, mRNAs and lipids, which could be potential biomarkers.
Materials and Methods: To evaluate the biomarker potential of the exosome derived RNA and protein content, we first optimized an extraction protocol for exosomes in plasma based on the Optiprep density gradient protocol. After purification, the exosomes are characterized and quantified using Western Blot and Nanosight analysis. Later, the RNA and protein fraction of the extracted exosomes from 60 NSCLC, 60 pancreatic cancer and 60 colorectal cancer patients is compared to 60 healthy controls. The proteome content is evaluated using mass spectrometry based quantitative shotgun proteomics. The RNA profiles are generated using next generation sequencing. After profound bioinformatics analysis, the potential of the RNA mand protein profiles will be evaluated for diagnostic, prognostic and therapy purposes.
Results: We are able to purify and characterize exosome material from plasma samples derived from patients. The data from nanosight analysis and Western blot indicate that we are able to work with very pure exosome samples for RNA and protein axtraction. Currently, RNA and protein profiles from different cancer types are being compared to healthy controls.
Conclusion: The first step in bringing exosome analysis to the clinic is optimized: exosome purification and characterization. In a next step, RNA and protein profiles are being evaluated as potential biomarkers.

3.2257 RacGTPase-activating protein 1 interacts with hepatitis C virus polymerase NS5B to regulate viral replication

Wu, M-J., Ke, P-Y. and Horng, J-T.
Biochem. Biophys. Res. Comm., ***454****, 19-24 (2014)*

Hepatitis C virus (HCV) is a positive-strand RNA virus responsible for chronic liver disease and hepatocellular carcinoma (HCC). RacGTPase-activating protein 1 (RacGAP1) plays an important role during GTP hydrolysis to GDP in Rac1 and CDC42 protein and has been demonstrated to be upregulated in several cancers, including HCC. However, the molecular mechanism leading to the upregulation of RacGAP1 remains poorly understood. Here, we showed that RacGAP1 levels were enhanced in HCV cell-culture-derived (HCVcc) infection. More importantly, we illustrated that RacGAP1 interacts with the viral protein NS5B in mammalian cells. The small interfering RNA (siRNA)-mediated knockdown of RacGAP1 in human hepatoma cell lines inhibited replication of HCV RNA, protein, and production of infectious particles of HCV genotype 2a strain JFH1. Conversely, these were reversed by the expression of a siRNA-resistant RacGAP1 recombinant protein. In addition, viral protein NS5B polymerase activity was significantly reduced by silencing RacGAP1 and, vice versa, was increased by overexpression of RacGAP1 in a cell-based reporter assay. Our results suggest that RacGAP1 plays a crucial role in HCV replication by affecting viral protein NS5B polymerase activity and holds importance for antiviral drug development.

3.2258 Endonuclease G preferentially cleaves 5-hydroxymethylcytosine-modified DNA creating a substrate for recombination

Robertson, A.B., Robertson, J., Fusser, M. and Klungland, A.
Nucleic Acids Res., ***42(21)****, 13280-13293 (2014)*

5-hydroxymethylcytosine (5hmC) has been suggested to be involved in various nucleic acid transactions and cellular processes, including transcriptional regulation, demethylation of 5-methylcytosine and stem cell pluripotency. We have identified an activity that preferentially catalyzes the cleavage of double-stranded 5hmC-modified DNA. Using biochemical methods we purified this activity from mouse liver extracts and demonstrate that the enzyme responsible for the cleavage of 5hmC-modified DNA is Endonuclease G (EndoG). We show that recombinant EndoG preferentially recognizes and cleaves a core sequence when one specific cytosine within that core sequence is hydroxymethylated. Additionally, we

provide *in vivo* evidence that EndoG catalyzes the formation of double-stranded DNA breaks and that this cleavage is dependent upon the core sequence, EndoG and 5hmC. Finally, we demonstrate that the 5hmC modification can promote conservative recombination in an EndoG-dependent manner.

3.2259 Cyclopamine Modulates {gamma}-Secretase-mediated Cleavage of Amyloid Precursor Protein by Altering Its Subcellular Trafficking and Lysosomal Degradation
Vorobyeva, A.G., Lee, r., Miller, S., Longren, C., Sharoni, M., Kandelwal, P.J. and Saunders, A.J.
J. Biol. Chem., ***289(48)***, *33258-33274 (2014)*

Alzheimer disease (AD) is a progressive neurodegenerative disease leading to memory loss. Numerous lines of evidence suggest that amyloid-β (Aβ), a neurotoxic peptide, initiates a cascade that results in synaptic dysfunction, neuronal death, and eventually cognitive deficits. Aβ is generated by the proteolytic processing of the amyloid precursor protein (APP), and alterations to this processing can result in Alzheimer disease. Using *in vitro* and *in vivo* models, we identified cyclopamine as a novel regulator of γ-secretase-mediated cleavage of APP. We demonstrate that cyclopamine decreases Aβ generation by altering APP retrograde trafficking. Specifically, cyclopamine treatment reduced APP-C-terminal fragment (CTF) delivery to the *trans*-Golgi network where γ-secretase cleavage occurs. Instead, cyclopamine redirects APP-CTFs to the lysosome. These data demonstrate that cyclopamine treatment decreases γ-secretase-mediated cleavage of APP. In addition, cyclopamine treatment decreases the rate of APP-CTF degradation. Together, our data demonstrate that cyclopamine alters APP processing and Aβ generation by inducing changes in APP subcellular trafficking and APP-CTF degradation.

3.2260 BPIFB3 Regulates Autophagy and Coxsackievirus B Replication through a Noncanonical Pathway Independent of the Core Initiation Machinery
Delorme-Axford, E., Morosky, S., Bomberger, J., Stolz, D.B., Jackson, W.T. and Coyne, C.B.
mBio, ***5(6)***, *e02147 (2014)*

Enteroviruses require autophagy to facilitate the formation of autophagosome (AP)-like double-membrane vesicles that provide the scaffolding for RNA replication. Here, we identify bactericidal/permeability-increasing protein (BPI) fold-containing family B, member 3 (BPIFB3) as a gene whose silencing greatly enhances coxsackievirus B (CVB) replication and induces dramatic alterations in the morphology of CVB-induced replication organelles. We show that BPIFB3 is associated with the endoplasmic reticulum (ER), and its silencing by RNA interference enhances basal levels of autophagy and promotes increased autophagy during CVB replication. Conversely, overexpression of BPIFB3 inhibits CVB replication, dramatically alters the morphology of LC3B-positive vesicles, and suppresses autophagy in response to rapamaycin. In addition, we found that, whereas silencing of core autophagy components associated with the initiation of APs in control cells suppressed CVB replication, silencing of these same components had no effect on CVB-induced autophagy or viral replication in cells transfected with BPIFB3 small interfering RNA. Based on these results, taken together, this study reports on a previously uncharacterized regulator of enterovirus infection that controls replication through a noncanonical pathway independent from the core autophagy initiation machinery.

3.2261 Isolation and identification of membrane vesicle-associated proteins in Gram-positive bacteria and mycobacteria
Prados-Rosales, R., Brown, L., Casadevall, A., Montalvo-Qiros, S. and Luque-garcia, J.L.
MethodsX, ***1***, *124-129 (2014)*

Many intracellular bacterial pathogens naturally release membrane vesicles (MVs) under a variety of growth environments. For pathogenic bacteria there are strong evidences that released MVs are a delivery mechanism for the release of immunologically active molecules that contribute to virulence. Identification of membrane vesicle-associated proteins that can act as immunological modulators is crucial for opening up new horizons for understanding the pathogenesis of certain bacteria and for developing novel vaccines. In this protocol, we provide all the details for isolating MVs secreted by either mycobacteria or Gram-positive bacteria and for the subsequent identification of the protein content of the MVs by mass spectrometry. The protocol is adapted from Gram-negative bacteria and involves four main steps: (1) isolation of MVs from the culture media; (2) purification of MVs by density gradient ultrucentrifugation; (3) acetone precipitation of the MVs protein content and in-solution trypsin digestion and (4) mass spectrometry analysis of the generated peptides and protein identification. Our modifications are: Growing Mycobacteria in a chemically defined media to reduce the number of unrelated bacterial components in the supernatant.

The use of an ultrafiltration system, which allows concentrating larger volumes.
In solution digestion of proteins followed by peptides purification by ziptip.

3.2262 Exosome-associated hepatitis C virus in cell cultures and patient plasma
Liu, Z., Zhang, X., Yu, Q. and He, J.J.
Biochem. Biophys. Res. Comm., ***455***, *218-222 (2014)*

Hepatitis C virus (HCV) infects its target cells in the form of cell-free viruses and through cell–cell contact. Here we report that HCV is associated with exosomes. Using highly purified exosomes and transmission electron microscopic imaging, we demonstrated that HCV occurred in both exosome-free and exosome-associated forms. Exosome-associated HCV was infectious and resistant to neutralization by an anti-HCV neutralizing antibody. There were more exosome-associated HCV than exosome-free HCV detected in the plasma of HCV-infected patients. These results suggest exosome-associated HCV as an alternative form for HCV infection and transmission.

3.2263 Soluble adenylyl cyclase of sea urchin spermatozoa
Vacquier, V.D., Loza-Huerta, A., Garcia-Rincon, J., Darszon, A. and Beltran, C.
Biochim. Biophys. Acta, ***1842***, *2621-2628 (2014)*

Fertilization, a key step in sexual reproduction, requires orchestrated changes in cAMP concentrations. It is notable that spermatozoa (sperm) are among the cell types with extremely high adenylyl cyclase (AC) activity. As production and consumption of this second messenger need to be locally regulated, the discovery of soluble AC (sAC) has broadened our understanding of how such cells deal with these requirements. In addition, because sAC is directly regulated by HCO_3^- it is able to translate CO_2/HCO_3^-/pH changes into cAMP levels. Fundamental sperm functions such as maturation, motility regulation and the acrosome reaction are influenced by cAMP; this is especially true for sperm of the sea urchin (SU), an organism that has been a model in the study of fertilization for more than 130 years. Here we summarize the discovery and properties of SU sperm sAC, and discuss its involvement in sperm physiology. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

3.2264 Phosphatidylinositol 3-kinase and COPII generate LC3 lipidation vesicles from the ER-Golgi intermediate compartment
Ge, L., Zhang, M. and Scheckman, R.
eLife, ***3***, *e04135 (2014)*

Formation of the autophagosome requires significant membrane input from cellular organelles. However, no direct evidence has been developed to link autophagic factors and the mobilization of membranes to generate the phagophore. Previously, we established a cell-free LC3 lipidation reaction to identify the ER-Golgi intermediate compartment (ERGIC) as a membrane source for LC3 lipidation, a key step of autophagosome biogenesis (Ge et al., eLife 2013; 2:e00947). We now report that starvation activation of autophagic phosphotidylinositol-3 kinase (PI3K) induces the generation of small vesicles active in LC3 lipidation. Subcellular fractionation studies identified the ERGIC as the donor membrane in the generation of small lipidation-active vesicles. COPII proteins are recruited to the ERGIC membrane in starved cells, dependent on active PI3K. We conclude that starvation activates the autophagic PI3K, which in turn induces the recruitment of COPII to the ERGIC to bud LC3 lipidation-active vesicles as one potential membrane source of the autophagosome. - See more at:
http://elifesciences.org/content/3/e04135.abstract?maxtoshow=&HITS=10&hits=150&RESULTFORMAT=1&andorexacttitle=and&andorexacttitleabs=and&fulltext=Optiprep%20or%20iodixanol&andorexactfulltext=and&searchid=1&FIRSTINDEX=0&sortspec=date&fdate=1/1/2014&tdate=12/31/2014&resourcetype=HWCIT#sthash.8dxaTbFc.dpuf

3.2265 Lenalidomide Induces Lipid Raft Assembly to Enhance Erythropoietin Receptor Signaling in Myelodysplastic Syndrome Progenitors
McGraw, K.L., Basiiorka, A.A., Johnson, J.O., Clark, J., Caceres, G., Padron, E., Heaton, R., Ozawa, Y., Wei, S., Sokol, L. and List, A.F.
PloS One, ***9(12)***, *e11429 (2014)*

Anemia remains the principal management challenge for patients with lower risk Myelodysplastic Syndromes (MDS). Despite appropriate cytokine production and cellular receptor display, erythropoietin

receptor (EpoR) signaling is impaired. We reported that EpoR signaling is dependent upon receptor localization within lipid raft microdomains, and that disruption of raft integrity abolishes signaling capacity. Here, we show that MDS erythroid progenitors display markedly diminished raft assembly and smaller raft aggregates compared to normal controls ($p = 0.005$, raft number; $p = 0.023$, raft size). Because lenalidomide triggers raft coalescence in T-lymphocytes promoting immune synapse formation, we assessed effects of lenalidomide on raft assembly in MDS erythroid precursors and UT7 cells. Lenalidomide treatment rapidly induced lipid raft formation accompanied by EpoR recruitment into raft fractions together with STAT5, JAK2, and Lyn kinase. The JAK2 phosphatase, CD45, a key negative regulator of EpoR signaling, was displaced from raft fractions. Lenalidomide treatment prior to Epo stimulation enhanced both JAK2 and STAT5 phosphorylation in UT7 and primary MDS erythroid progenitors, accompanied by increased STAT5 DNA binding in UT7 cells, and increased erythroid colony forming capacity in both UT7 and primary cells. Raft induction was associated with F-actin polymerization, which was blocked by Rho kinase inhibition. These data indicate that deficient raft integrity impairs EpoR signaling, and provides a novel strategy to enhance EpoR signal fidelity in non-del(5q) MDS.

3.2266 Egr-1 Activation by Cancer-Derived Extracellular Vesicles Promotes Endothelial Cell Migration via ERK1/2 and JNK Signaling Pathways
Yoon, Y.J., Kim, D-K., Yoon, C.M., Park, J., Kim, Y-K., Roh, T-Y and Gho, Y.S:
*PloS One, **9(12)**, e115170 (2014)*

Various mammalian cells, including cancer cells, shed extracellular vesicles (EVs), also known as exosomes and microvesicles, into surrounding tissues. These EVs play roles in tumor growth and metastasis by promoting angiogenesis. However, the detailed mechanism of how cancer-derived EVs elicit endothelial cell activation remains unknown. Here, we provide evidence that early growth response-1 (Egr-1) activation in endothelial cells is involved in the angiogenic activity of colorectal cancer cell-derived EVs. Both RNA interference–mediated downregulation of Egr-1 and ERK1/2 or JNK inhibitor significantly blocked EV-mediated Egr-1 activation and endothelial cell migration. Furthermore, lipid raft-mediated endocytosis inhibitor effectively blocked endothelial Egr-1 activation and migration induced by cancer-derived EVs. Our results suggest that Egr-1 activation in endothelial cells may be a key mechanism involved in the angiogenic activity of cancer-derived EVs. These findings will improve our understanding regarding the proangiogenic activities of EVs in diverse pathological conditions including cancer, cardiovascular diseases, and neurodegenerative diseases.

3.2267 Proteomic Analysis of the Acidocalcisome, an Organelle Conserved from Bacteria to Human Cells
Huang, G., Ulrich, P.N., Storey, M., Johnson, D., Tischer, J., Tovar, J.A., Moreno, S.N.J., Orlando, R. and Docompo, R.
*Plos Pathogens, **10(12)**, e1004555 (2014)*

Acidocalcisomes are acidic organelles present in a diverse range of organisms from bacteria to human cells. In this study acidocalcisomes were purified from the model organism *Trypanosoma brucei*, and their protein composition was determined by mass spectrometry. The results, along with those that we previously reported, show that acidocalcisomes are rich in pumps and transporters, involved in phosphate and cation homeostasis, and calcium signaling. We validated the acidocalcisome localization of seven new, putative, acidocalcisome proteins (phosphate transporter, vacuolar H^+-ATPase subunits *a* and *d*, vacuolar iron transporter, zinc transporter, polyamine transporter, and acid phosphatase), confirmed the presence of six previously characterized acidocalcisome proteins, and validated the localization of five novel proteins to different subcellular compartments by expressing them fused to epitope tags in their endogenous loci or by immunofluorescence microscopy with specific antibodies. Knockdown of several newly identified acidocalcisome proteins by RNA interference (RNAi) revealed that they are essential for the survival of the parasites. These results provide a comprehensive insight into the unique composition of acidocalcisomes of *T. brucei*, an important eukaryotic pathogen, and direct evidence that acidocalcisomes are especially adapted for the accumulation of polyphosphate.

3.2268 New insights in the composition of extracellular vesicles from pancreatic cancer cells: implications for biomarkers and functions
Klein-Scory, S., Tehrani, M.M., Eilert-Micus, C., Adamczyk, K.A., Wojtalewics, N., Schnölzer, M., Hahn, S.A., Schmiegel, Wa. And Schwarte-Waldhoff, I.
*Proteome Science, **12**:50 (2014)*

Background

Pancreatic cancer development is associated with characteristic alterations like desmoplastic reaction and immune escape which are mediated by the cell-cell communication mechanism and by the microenvironment of the cells. The whole of released components are important determinants in these processes. Especially the extracellular vesicles released by pancreatic cancer cells play a role in cell communication and modulate cell growth and immune responses.
Results
Here, we present the proteomic description of affinity purified extracellular vesicles from pancreatic tumour cells, compared to the secretome, defined as the whole of the proteins released by pancreatic cancer cells. The proteomic data provide comprehensive catalogues of hundreds of proteins, and the comparison reveals a special proteomic composition of pancreatic cancer cell derived extracellular vesicles. The functional analysis of the protein composition displayed that membrane proteins, glycoproteins, small GTP binding proteins and a further, heterogeneous group of proteins are enriched in vesicles, whereas proteins derived from proteasomes and ribosomes, as well as metabolic enzymes, are not components of the vesicles. Furthermore proteins playing a role in carcinogenesis and modulators of the extracellular matrix (ECM) or cell-cell interactions are components of affinity purified extracellular vesicles.
Conclusion
The data deepen the knowledge of extracellular vesicle composition by hundreds of proteins that have not been previously described as vesicle components released by pancreatic cancer cells. Extracellular vesicles derived from pancreatic cancer cells show common proteins shared with other vesicles as well as cell type specific proteins indicating biomarker candidates and suggesting functional roles in cancer cell stroma interactions.

3.2269 Stable Cell Surface Expression of GPI-Anchored Proteins, but not Intracellular Transport, Depends on their Fatty Acid Structure
Jaensch, N., Correa, I.R. and Watanabe, R.
Traffic, ***15(12)***, *1305-1329 (2014)*

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are a class of lipid anchored proteins expressed on the cell surface of eukaryotes. The potential interaction of GPI-APs with ordered lipid domains enriched in cholesterol and sphingolipids has been proposed to function in the intracellular transport of these lipid anchored proteins. Here, we examined the biological importance of two saturated fatty acids present in the phosphatidylinositol moiety of GPI-APs. These fatty acids are introduced by the action of lipid remodeling enzymes and required for the GPI-AP association within ordered lipid domains. We found that the fatty acid remodeling is not required for either efficient Golgi-to-plasma membrane transport or selective endocytosis via GPI-enriched early endosomal compartment (GEEC)/ clathrin-independent carrier (CLIC) pathway, whereas cholesterol depletion significantly affects both pathways independent of their fatty acid structure. Therefore, the mechanism of cholesterol dependence does not appear to be related to the interaction with ordered lipid domains mediated by two saturated fatty acids. Furthermore, cholesterol extraction drastically releases the unremodeled GPI-APs carrying an unsaturated fatty acid from the cell surface, but not remodeled GPI-APs carrying two saturated fatty acids. This underscores the essential role of lipid remodeling to ensure a stable membrane association of GPI-APs particularly under potential membrane lipid perturbation.

3.2270 Mammalian CORVET Is Required for Fusion and Conversion of Distinct Early Endosome Subpopulations
Perini, E.D., Schaefer, R., Stöter, M., Kalaidzidis, Y. and Zerial, M.
Traffic, ***15(12)***, *1366-1389 (2014)*

Early endosomes are organized in a network of vesicles shaped by cycles of fusion, fission, and conversion to late endosomes. In yeast, endosome fusion and conversion are regulated, among others, by CORVET, a hexameric protein complex. In the mammalian endocytic system, distinct subpopulations of early endosomes labelled by the Rab5 effectors APPL1 and EEA1 are present. Here, the function of mammalian CORVET with respect to these endosomal subpopulations was investigated. Tgfbrap1 as CORVET-specific subunit and functional ortholog of Vps3p was identified, demonstrating that it is differentially distributed between APPL1 and EEA1 endosomes. Surprisingly, depletion of CORVET-specific subunits caused fragmentation of APPL1-positive endosomes but not EEA1 endosomes *in vivo*. These and *in vitro* data suggest that CORVET plays a role in endosome fusion independently of EEA1. Depletion of CORVET subunits caused accumulation of large EEA1 endosomes indicative of another role in the conversion of EEA1 endosomes into late endosomes. In addition, depletion of CORVET-specific subunits caused alterations in transport depending on both the type of cargo and the specific endosomal

subpopulation. These results demonstrate that CORVET plays distinct roles at multiple stages in the mammalian endocytic pathway.

3.2271 **Effects of heat shock protein 90 expression on pectoralis major oxidation in broilers exposed to acute heat stress**
Hao, Y. and Gu, X.H.
*Poultry Science, **93(11)**, 2709-2717 (2014)*

This study was conducted to determine the effects of heat shock protein 90 (HSP90) expression on pH, lipid peroxidation, heat shock protein 70 (HSP70), and glucocorticoid receptor (GR) expression of pectoralis major in broilers exposed to acute heat stress. In total, 90 male broilers were randomly allocated to 3 groups: control (CON), heat stress (HS), or geldanamycin treatment (GA). On d 41, the broilers in the GA group were injected intraperitoneally with GA (5 μg/kg of BW), and the broilers in the CON and HS groups were injected intraperitoneally with saline. Twenty-four hours later, the broilers in the CON group were moved to environmental chambers controlled at 22°C for 2 h, and the broilers in the HS and GA groups were moved to environmental chambers controlled at 40°C for 2 h. The pH values of the pectoralis major after 30 min and 24 h of chilling after slaughter of HS and GA broilers were significantly lower ($P < 0.01$) than those of the CON broilers. Heat stress caused significant increases in sera corticosterone and lactic dehydrogenase, the activity of malondialdehyde and superoxide dismutase, the expression of HSP90 and HSP70, and nuclear expression of GR protein in the pectoralis major ($P < 0.05$). Heat stress induced a significant decrease in GR protein expression in the cytoplasm and GR mRNA expression. Furthermore, the low expression of HSP90 significantly increased levels of lactic dehydrogenase and malondialdehyde and GR protein expression in the cytoplasm under heat stress ($P < 0.01$), and significantly decreased nuclear GR protein expression ($P < 0.01$). Heat shock protein 90 was positively correlated with corticosterone and superoxide dismutase activities ($P < 0.01$), and HSP90 mRNA was negatively correlated with pH after chilling for 24 h. The results demonstrated that HSP90 plays a pivotal role in protecting cells from oxidation.

3.2272 **The ESCRT machinery regulates the secretion and long-range activity of Hedgehog**
Matusek, T., Wendler, F., Poles, S., Pizette, S., D'Angelo, G., Fürthauer, M. and Therond, P.P
*Nature, **516**, 99-103 (2014)*

The conserved family of Hedgehog (Hh) proteins acts as short- and long-range secreted morphogens, controlling tissue patterning and differentiation during embryonic development[1]. Mature Hh carries hydrophobic palmitic acid and cholesterol modifications essential for its extracellular spreading[2]. Various extracellular transportation mechanisms for Hh have been suggested, but the pathways actually used for Hh secretion and transport *in vivo* remain unclear. Here we show that Hh secretion in *Drosophila* wing imaginal discs is dependent on the endosomal sorting complex required for transport (ESCRT)[3]. *In vivo* the reduction of ESCRT activity in cells producing Hh leads to a retention of Hh at the external cell surface. Furthermore, we show that ESCRT activity in Hh-producing cells is required for long-range signalling. We also provide evidence that pools of Hh and ESCRT proteins are secreted together into the extracellular space *in vivo* and can subsequently be detected together at the surface of receiving cells. These findings uncover a new function for ESCRT proteins in controlling morphogen activity and reveal a new mechanism for the transport of secreted Hh across the tissue by extracellular vesicles, which is necessary for long-range target induction.

3.2273 **The emerging role of extracellular vesicles as biomarkers for urogenital cancers**
Nawaz, M., Camussi, G., Valadi, H., Nazarenko, I., Ekström, K., Wang, X., Principe, S., Shah, N., Ashraf, N.M., Fatima, F., Neder, L. and Kislinger, T.
*Nat. Rev. Urol., **11**, 688-701 (2014)*

The knowledge gained from comprehensive profiling projects that aim to define the complex genomic alterations present within cancers will undoubtedly improve our ability to detect and treat those diseases, but the influence of these resources on our understanding of basic cancer biology is still to be demonstrated. Extracellular vesicles have gained considerable attention in past years, both as mediators of intercellular signalling and as potential sources for the discovery of novel cancer biomarkers. In general, research on extracellular vesicles investigates either the basic mechanism of vesicle formation and cargo incorporation, or the isolation of vesicles from available body fluids for biomarker discovery. A deeper understanding of the cargo molecules present in extracellular vesicles obtained from patients with urogenital cancers,

through high-throughput proteomics or genomics approaches, will aid in the identification of novel diagnostic and prognostic biomarkers, and can potentially lead to the discovery of new therapeutic targets.

3.2274 Effects of sildenafil on nanostructural and nanomechanical changes in mitochondria in an ischaemia-reperfusion rat model

Lee, K.H., Kwon, S.J., Woo, J-S., Lee, G-J., Lee, S-R., jang, H-H., Kim, H.S., Kim, J.W., Park, H.K., Cho, K.S. and Kim, W.

*Clinical and Experimental Pharmacology and Physiology, **41(10)**, 763-768 (2014)*

Sildenafil exerts cardioprotective effects by activating the opening of mitochondrial ATP-sensitive potassium channels to attenuate ischaemia–reperfusion (IR) injury. In the present study, we used atomic force microscopy (AFM) to investigate changes in mitochondrial morphology and properties to assess sildenafil-mediated cardioprotection in a rat myocardial infarction model. To investigate the cardioprotective effects of sildenafil, we used an *in vivo* Sprague-Dawley rat model of IR. Rats were randomly divided into three groups: (i) sham-operated rats (control; $n = 5$); (ii) IR-injured rats treated with vehicle (normal saline; IR; $n = 10$); and (iii) IR-injured rats treated with 0.75 mg/kg, i.p., sildenafil (IR + Sil; $n = 10$). Morphological and mechanical changes to mitochondria were analysed by AFM. Infarct areas were significantly reduced in sildenafil-treated rats (7.8 ± 3.9% *vs* 20.4 ± 7.0% in the sildenafil-treated and untreated IR groups, respectively; relative reduction 62%; $P < 0.001$). Analysis of mitochondria by AFM showed that IR injury significantly increased the areas of isolated mitochondria compared with control (24 150 ± 18 289 *vs* 1495 ± 1139 nm^2, respectively; $P < 0.001$), indicative of mitochondrial swelling. Pretreatment with sildenafil before IR injury reduced the mitochondrial areas (7428 ± 3682 nm^2; $P < 0.001$; relative reduction 69.2% compared with the IR group) and ameliorated the adhesion force of mitochondrial surfaces. Together, these results suggest that sildenafil has cardioprotective effects against IR injury in a rat model by improving the morphological and mechanical characteristics of mitochondria.

3.2275 A Golgi-Localized Pool of the Mitotic Checkpoint Component Mad1 Controls Integrin Secretion and Cell Migration

Wan, J., Zhu, F., Zasadil, L.M., Yu, J., Wang, L., Johnson, A., Berthier, E., Beebe, D.J., Audhya, A. and Weaver, B.A.

*Current Biology, **24(22)**, 2687-2692 (2014)*

Mitotic arrest deficient 1 (Mad1) plays a well-characterized role in the major cell-cycle checkpoint that regulates chromosome segregation during mitosis, the mitotic checkpoint (also known as the spindle assembly checkpoint). During mitosis, Mad1 recruits Mad2 to unattached kinetochores [1, 2], where Mad2 is converted into an inhibitor of the anaphase-promoting complex/cyclosome bound to its specificity factor, Cdc20 [1, 3–6]. During interphase, Mad1 remains tightly bound to Mad2 [2, 3, 7, 8], and both proteins localize to the nucleus and nuclear pores [9, 10], where they interact with Tpr (translocated promoter region). Recently, it has been shown that interaction with Tpr stabilizes both proteins [11] and that Mad1 binding to Tpr permits Mad2 to associate with Cdc20 [12]. However, interphase functions of Mad1 that do not directly affect the mitotic checkpoint have remained largely undefined. Here we identify a previously unrecognized interphase distribution of Mad1 at the Golgi apparatus. Mad1 colocalizes with multiple Golgi markers and cosediments with Golgi membranes. Although Mad1 has previously been thought to constitutively bind Mad2, Golgi-associated Mad1 is Mad2 independent. Depletion of Mad1 impairs secretion of α5 integrin and results in defects in cellular attachment, adhesion, and FAK activation. Additionally, reduction of Mad1 impedes cell motility, while its overexpression accelerates directed cell migration. These results reveal an unexpected role for a mitotic checkpoint protein in secretion, adhesion, and motility. More generally, they demonstrate that, in addition to generating aneuploidy, manipulation of mitotic checkpoint genes can have unexpected interphase effects that influence tumor phenotypes.

3.2276 Aberrant Glycosylation and Localization of Polycystin-1 Cause Polycystic Kidney in an AQP11 Knockout Model

Inoue, Y., Sohara, E., Kobayashi, K., Chiga, M., Rai, T., Ishibashi, K., Horie, S., Su, X., Zhou, J., Sasaki, S. and Uchida, S.

*J. Am. Soc. Nephrol., **25**, 2789-2799 (2014)*

We previously reported that disruption of the aquaporin-11 (AQP11) gene in mice resulted in cystogenesis in the kidney. In this study, we aimed to clarify the mechanism of cystogenesis in AQP11(−/−) mice. To enable the analyses of AQP11 at the protein level *in vivo*, AQP11 BAC transgenic mice (Tg^{AQP11}) that

express 3×HA-tagged AQP11 protein were generated. This AQP11 localized to the endoplasmic reticulum (ER) of proximal tubule cells in Tg^{AQP11} mice and rescued renal cystogenesis in AQP11(−/−) mice. Therefore, we hypothesized that the absence of AQP11 in the ER could result in impaired quality control and aberrant trafficking of polycystin-1 (PC-1) and polycystin-2 (PC-2). Compared with kidneys of wild-type mice, AQP11(−/−) kidneys exhibited increased protein expression levels of PC-1 and decreased protein expression levels of PC-2. Moreover, PC-1 isolated from AQP11(−/−) mice displayed an altered electrophoretic mobility caused by impaired *N*-glycosylation processing, and density gradient centrifugation of kidney homogenate and *in vivo* protein biotinylation revealed impaired membrane trafficking of PC-1 in these mice. Finally, we showed that the Pkd1(+/−) background increased the severity of cystogenesis in AQP11(−/−) mouse kidneys, indicating that PC-1 is involved in the mechanism of cystogenesis in AQP11(−/−) mice. Additionally, the primary cilia of proximal tubules were elongated in AQP11(−/−) mice. Taken together, these data show that impaired glycosylation processing and aberrant membrane trafficking of PC-1 in AQP11(−/−) mice could be a key mechanism of cystogenesis in AQP11(−/−) mice.

3.2277 Dual Proteolytic Pathways Govern Glycolysis and Immune Competence
Lu, W. et al
*Cell, **159**, 1578-1590 (2014)*

Proteasomes and lysosomes constitute the major cellular systems that catabolize proteins to recycle free amino acids for energy and new protein synthesis. Tripeptidyl peptidase II (TPPII) is a large cytosolic proteolytic complex that functions in tandem with the proteasome-ubiquitin protein degradation pathway. We found that autosomal recessive *TPP2* mutations cause recurrent infections, autoimmunity, and neurodevelopmental delay in humans. We show that a major function of TPPII in mammalian cells is to maintain amino acid levels and that TPPII-deficient cells compensate by increasing lysosome number and proteolytic activity. However, the overabundant lysosomes derange cellular metabolism by consuming the key glycolytic enzyme hexokinase-2 through chaperone-mediated autophagy. This reduces glycolysis and impairs the production of effector cytokines, including IFN-γ and IL-1β. Thus, TPPII controls the balance between intracellular amino acid availability, lysosome number, and glycolysis, which is vital for adaptive and innate immunity and neurodevelopmental health.

3.2278 Sirtuin 4 Is a Lipoamidase Regulating Pyruvate Dehydrogenase Complex Activity
Mathias, R., Greco, T.M., Oberstein, A., Budayeva, H.G., Chakrabarti, R., Rowland, E.A., Kang, Y., Shenk, T. and Cristea, I.M.
*Cell, **159**, 1615-1625 (2014)*

Sirtuins (SIRTs) are critical enzymes that govern genome regulation, metabolism, and aging. Despite conserved deacetylase domains, mitochondrial SIRT4 and SIRT5 have little to no deacetylase activity, and a robust catalytic activity for SIRT4 has been elusive. Here, we establish SIRT4 as a cellular lipoamidase that regulates the pyruvate dehydrogenase complex (PDH). Importantly, SIRT4 catalytic efficiency for lipoyl- and biotinyl-lysine modifications is superior to its deacetylation activity. PDH, which converts pyruvate to acetyl-CoA, has been known to be primarily regulated by phosphorylation of its E1 component. We determine that SIRT4 enzymatically hydrolyzes the lipoamide cofactors from the E2 component dihydrolipoyllysine acetyltransferase (DLAT), diminishing PDH activity. We demonstrate SIRT4-mediated regulation of DLAT lipoyl levels and PDH activity in cells and in vivo, in mouse liver. Furthermore, metabolic flux switching via glutamine stimulation induces SIRT4 lipoamidase activity to inhibit PDH, highlighting SIRT4 as a guardian of cellular metabolism.

3.2279 An AUTS2–Polycomb complex activates gene expression in the CNS
Gao, Z., Lee, P., Stafford, J.M., von Schimmelmann, M., Schaefer, A. and Reinberg, D.
*Nature, **516**, 349-354 (2014)*

Naturally occurring variations of Polycomb repressive complex 1 (PRC1) comprise a core assembly of Polycomb group proteins and additional factors that include, surprisingly, autism susceptibility candidate 2 (AUTS2). Although AUTS2 is often disrupted in patients with neuronal disorders, the mechanism underlying the pathogenesis is unclear. We investigated the role of AUTS2 as part of a previously identified PRC1 complex (PRC1–AUTS2), and in the context of neurodevelopment. In contrast to the canonical role of PRC1 in gene repression, PRC1–AUTS2 activates transcription. Biochemical studies demonstrate that the CK2 component of PRC1–AUTS2 neutralizes PRC1 repressive activity, whereas AUTS2-mediated recruitment of P300 leads to gene activation. Chromatin immunoprecipitation followed

by sequencing (ChIP-seq) demonstrated that AUTS2 regulates neuronal gene expression through promoter association. Conditional targeting of Auts2 in the mouse central nervous system (CNS) leads to various developmental defects. These findings reveal a natural means of subverting PRC1 activity, linking key epigenetic modulators with neuronal functions and diseases.

Characterization of physical properties of tissue factor-containing microvesicles and a comparison of ultracentrifuge-based recovery procedures
Ettelaie, C., Collier, M.E.W., Maraveyas, A. and Ettelaie, R.
*J. Extracellular Vesicles, **3**:23592 (2014)*

Microvesicles were isolated from the conditioned media of 3 cell lines (MDA-MB-231, AsPC-1 and A375) by ultracentrifugation at a range of relative centrifugal forces, and the tissue factor (TF) protein and activity, microvesicle number, size distribution and relative density compared. Also, by expressing TF-tGFP in cells and isolating the microvesicles, the relative density of TF-containing microvesicles was established. Nanoparticle tracking analysis (NTA) indicated that the larger-diameter microvesicles (>200 nm) were primarily sedimented at 100,000*g* and possessed TF-dependent thrombin and factor Xa generation potential, while in the absence of factor VII, all microvesicles possessed some thrombin generation capacity. Immuno-precipitation of TF-containing microvesicles followed by NTA also indicated the range of these microvesicles to be 200–400 nm. Analysis of the microvesicles by gradient density centrifugation showed that lower-density (<1.1 g/ml) microvesicles were mainly present in the samples recovered at 100,000*g* and were associated with TF antigen and activity. Analysis of these fractions by NTA confirmed that these fractions were principally composed of the larger-diameter microvesicles. Similar analysis of microvesicles from healthy or patient plasma supported those obtained from conditioned media indicating that TF activity was mainly associated with lower-density microvesicles. Furthermore, centrifugation of healthy plasma, supplemented with TF-tGFP-containing microvesicles, resulted in 67% retrieval of the fluorescent microvesicles at 100,000*g*, but only 26% could be recovered at 20,000*g*. Pre-centrifugation of conditioned media or plasma at 10,000*g* improved the speed and yield of recovered TF-containing microvesicles by subsequent centrifugation at either 20,000*g* or 100,000*g*. In conclusion, TF appears to be associated with low-density (1.03–1.08 g/ml), larger-diameter (200–350 nm) microvesicles.

The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling
Van Deun, J., Mestdagh, P., Sormunen, R., Cocquyt, V., Vermaelen, K., Vandesompele, J., Bracke, M., De Wever, O. and Hendrix, A.
*J. Extracellular Vesicles, **3**:24858 (2014)*

Despite an enormous interest in the role of extracellular vesicles, including exosomes, in cancer and their use as biomarkers for diagnosis, prognosis, drug response and recurrence, there is no consensus on dependable isolation protocols. We provide a comparative evaluation of 4 exosome isolation protocols for their usability, yield and purity, and their impact on downstream omics approaches for biomarker discovery. OptiPrep density gradient centrifugation outperforms ultracentrifugation and ExoQuick and Total Exosome Isolation precipitation in terms of purity, as illustrated by the highest number of CD63-positive nanovesicles, the highest enrichment in exosomal marker proteins and a lack of contaminating proteins such as extracellular Argonaute-2 complexes. The purest exosome fractions reveal a unique mRNA profile enriched for translation, ribosome, mitochondrion and nuclear lumen function. Our results demonstrate that implementation of high purification techniques is a prerequisite to obtain reliable omics data and identify exosome-specific functions and biomarkers.

3.2280 Membrane actions of 1α,25(OH)2D3 are mediated by Ca2+/calmodulin-dependent protein kinase II in bone and cartilage cells
Doroudi, M., Plaisance, M.C., Boyan, B.D. and Schwarz, Z.
*J. Steroid Biochem. Mol. Biol., **145**, 65-74 (2015)*

1α,25$(OH)_2D_3$ regulates osteoblasts and chondrocytes via its membrane-associated receptor, protein disulfide isomerase A3 (Pdia3) in caveolae. 1α,25$(OH)_2D_3$ binding to Pdia3 leads to phospholipase-A_2 (PLA_2)-activating protein (PLAA) activation, stimulating cytosolic PLA_2 and resulting in prostaglandin E2 (PGE_2) release and PKCα activation, subsequently stimulating differentiation. However, how PLAA transmits the signal to $cPLA_2$ is unknown. Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) activation is required for PLA_2 activation in vascular smooth muscle cells, suggesting a similar role in 1α,25$(OH)_2D_3$-dependent signaling. The aim of the present study is to evaluate the roles of CaM and

CaMKII as mediators of 1α,25(OH)$_2$D$_3$-stimulated PLAA-dependent activation of cPLA$_2$ and PKCα, and downstream biological effects. The results indicated that 1α,25(OH)$_2$D$_3$ and PLAA-peptide increased CaMKII activity within 9 min. Silencing Cav-1, Pdia3 or Plaa in osteoblasts suppressed this effect. Similarly, antibodies against Plaa or Pdia3 blocked 1α,25(OH)$_2$D$_3$-dependent CaMKII. Caveolae disruption abolished activation of CaMKII by 1α,25(OH)$_2$D$_3$ or PLAA. CaMKII-specific and CaM-specific inhibitors reduced cPLA$_2$ and PKC activities, PGE$_2$ release and osteoblast maturation markers in response to 1α,25(OH)$_2$D$_3$. Camk2a-silenced but not Camk2b-silenced osteoblasts showed comparable effects. Immunoprecipitation showed increased interaction of CaM and PLAA in response to 1α,25(OH)$_2$D$_3$. The results indicate that membrane actions of 1α,25(OH)$_2$D$_3$ via Pdia3 triggered the interaction between PLAA and CaM, leading to dissociation of CaM from caveolae, activation of CaMKII, and downstream PLA$_2$ activation, and suggest that CaMKII plays a major role in membrane-mediated actions of 1α,25(OH)$_2$D$_3$.

3.2281 Fatty acid profiles from the plasma membrane and detergent resistant membranes of two plant species

Carmona-Salazar, L., El Hafidi, M., Gutierrez-Najera, N., Noyola-martinez, L., Gonzalez-Solis, A. and Gavilanes-Ruiz, M.
Phytochemistry, ***109***, *25-35 (2015)*

It is essential to establish the composition of the plant plasma membrane in order to understand its organization and behavior under continually changing environments. Knowledge of the lipid phase, in particular the fatty acid (FA) complex repertoire, is important since FAs determine many of the physical–chemical membrane properties. FAs are constituents of the membrane glycerolipid and sphingolipid backbones and can also be linked to some sterols. In addition, FAs are components of complex lipids that can constitute membrane micro-domains, and the use of detergent-resistant membranes is a common approach to study their composition. The diversity and cellular allocation of the membrane lipids containing FAs are very diverse and the approaches to analyze them provide only general information. In this work, a detailed FA analysis was performed using highly purified plasma membranes from bean leaves and germinating maize embryos and their respective detergent-resistant membrane preparations. The analyses showed the presence of a significant amount of very long chain FAs (containing 28C, 30C and 32C), in both plasma membrane preparations from bean and maize, that have not been previously reported. Herein is demonstrated that a significant enrichment of very long chain saturated FAs and saturated FAs can occur in detergent-resistant membrane preparations, as compared to the plasma membranes from both plant species. Considering that a thorough analysis of FAs is rarely performed in purified plasma membranes and detergent-resistant membranes, this work provides qualitative and quantitative evidence on the contributions of the length and saturation of FAs to the organization of the plant plasma membrane and detergent-resistant membranes.

3.2282 Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria

Klimentova, J. and Stulik, J.
Microbiol. Res., ***170***, *1-9 (2015)*

Abstract
Outer membrane vesicles secreted by gram-negative bacteria play an important role in bacterial physiology as well as in virulence and host–pathogen interaction. Isolated vesicles of some bacteria have also been studied for their immunomodulatory potential in the vaccine development. However, the production of vesicles in sufficient amount, purity and reproducibility remains a critical challenge for subsequent analyses in most bacteria. In the present review methods of production, isolation, purification and quantification of outer membrane vesicles are summarized and discussed.

3.2283 Downregulation of kinin B1 receptor function by B2 receptor heterodimerization and signaling

Zhang, X., Brovkovych, V., Zhang, Y., Tan, Ff. and Skidgel, R.A.
Cellular Signalling, ***27***, *90-103 (2015)*

Signaling through the G protein-coupled kinin receptors B1 (kB1R) and B2 (kB2R) plays a critical role in inflammatory responses mediated by activation of the kallikrein–kinin system. The kB2R is constitutively expressed and rapidly desensitized in response to agonist whereas kB1R expression is upregulated by inflammatory stimuli and it is resistant to internalization and desensitization. Here we show that the kB1R heterodimerizes with kB2Rs in co-transfected HEK293 cells and natively expressing endothelial cells, resulting in significant internalization and desensitization of the kB1R response in cells pre-treated with kB2R agonist. However, pre-treatment of cells with kB1R agonist did not affect subsequent kB2R

responses. Agonists of other G protein-coupled receptors (thrombin, lysophosphatidic acid) had no effect on a subsequent kB1R response. The loss of kB1R response after pretreatment with kB2R agonist was partially reversed with kB2R mutant Y129S, which blocks kB2R signaling without affecting endocytosis, or T342A, which signals like wild type but is not endocytosed. Co-endocytosis of the kB1R with kB2R was dependent on β-arrestin and clathrin-coated pits but not caveolae. The sorting pathway of kB1R and kB2R after endocytosis differed as recycling of kB1R to the cell surface was much slower than that of kB2R. In cytokine-treated human lung microvascular endothelial cells, pre-treatment with kB2R agonist inhibited kB1R-mediated increase in transendothelial electrical resistance (TER) caused by kB1R stimulation (to generate nitric oxide) and blocked the profound drop in TER caused by kB1R activation in the presence of pyrogallol (a superoxide generator). Thus, kB1R function can be downregulated by kB2R co-endocytosis and signaling, suggesting new approaches to control kB1R signaling in pathological conditions.

3.2284 **Design, synthesis and biological evaluation of novel pyrenyl derivatives as anticancer agents**
Bandyopadhyay, D., Sanchez, J.L., Guerrero, A.M., Chang, F-M., Granados, J.C., Short, J.D. and Banik, B.K.
Eur. J. Medicinal Chem., ***89***, *851-862 (2015)*

Polycyclic aromatic hydrocarbons are widespread in nature with a toxicity range from non-toxic to extremely toxic. A series of pyrenyl derivatives has been synthesized following a four-step strategy where the pyrene nucleus is attached with a basic heterocyclic moiety through a carbon linker. Virtual screening of the physicochemical properties and druggability has been carried out. The cytotoxicity of the compounds (**1**–**8**) have been evaluated in vitro against a small panel of human cancer cell lines which includes two liver cancer (HepG2 and Hepa 1–6), two colon cancer (HT-29 and Caco-2) and one each for cervical (HeLa) and breast (MCF-7) cancer cell lines. The IC_{50} data indicate that compound **6** and **8** are the most effective cytotoxic agents in the present set of pyrenyl derivatives, suggesting that having a 4-carbon linker is more effective than a 5-carbon linker and the presence of amide carbonyl groups in the linker severely reduces the efficacy of the compound. The compounds showed selectivity toward cancer cells at lower doses (<5 μM) when compared with the normal hepatocytes. The mechanism of action supports the cell death through apoptosis in a caspase-independent manner without cleavage of poly (ADP-ribose) polymerase (PARP), even though the compounds cause plasma membrane morphological changes. The compounds, whether highly cytotoxic or mildly cytotoxic, localize to the membrane of cells. The compounds with either a piperidine ring (**6**) or an N-methyl piperazine (**8**) in the side chain were both capable of circumventing the drug resistance in SKOV3-MDR1-M6/6 ovarian cancer cells overexpressing P-glycoprotein. Qualitative structure-activity relationship has also been studied.

3.2285 **Rotenone impairs autophagic flux and lysosomal functions in Parkinson's disease**
Wu, F., Xu, H.D., Guan, J.J., Hou, Y.S., Gu, J.H., Zhen, X.C. and Qin, Z.H.
Neuroscience, ***284***, *900-911 (2015)*

Background
Rotenone is an environmental neurotoxin that induces accumulation of α-synuclein and degeneration of dopaminergic neurons in substantia nigra pars compacta (SNpc), but the molecular mechanisms are not fully understood. We investigated whether rotenone induced impairment of autophagic flux and lysosomal functions.
Methods
Autophagy flux, accumulation of α-synuclein, lysosomal membrane integrity and neurodegeneration were assessed in the rotenone-treated rat model and PC12 cells, and the effects of the autophagy inducer trehalose on rotenone's cytotoxicity were also studied.
Results
Rotenone administration significantly reduced motor activity and caused a loss of tyrosine hydroxylase in SNpc of Lewis rats. The degeneration of nigral dopaminergic neurons was accompanied by the deposition of α-synuclein aggregates, autophagosomes and redistribution of cathepsin D from lysosomes to the cytosol. In cultured PC12 cells, rotenone also induced increases in protein levels of α-synuclein, microtubule-associated protein 1 light chain 3-II, Beclin 1, and p62. Rotenone increased lysosomal membrane permeability as evidenced by leakage of N-acetyl-beta-d-glucosaminidase and cathepsin D, the effects were blocked by reactive oxygen species scavenger tiron. Autophagy inducer trehalose enhanced the nuclear translocation of transcription factor EB, accelerated the clearance of autophagosomes and α-synuclein and attenuated rotenone-induced cell death of PC12 cells. Meanwhile, administration of trehalose to rats in drinking water (2%) decreased rotenone-induced dopaminergic neurons loss in SNpc.

Conclusions
These studies indicate that the lysosomal dysfunction contributes to rotenone's neurotoxicity and restoration of lysosomal function could be a new therapeutic strategy for Parkinson's disease.

3.2286 The Major Myelin-Resident Protein PLP Is Transported to Myelin Membranes via a Transcytotic Mechanism: Involvement of Sulfatide
Baron, W., Ozgen, H., Klunder, B., de Jonge, J.C., Nomden, A., Plat, A., Teifilieff, E., de Vries, H. and Hoekstra, D.
*Mol. Cell. Biol., **35(1)**, 288-302 (2015)*

Myelin membranes are sheet-like extensions of oligodendrocytes that can be considered membrane domains distinct from the cell's plasma membrane. Consistent with the polarized nature of oligodendrocytes, we demonstrate that transcytotic transport of the major myelin-resident protein proteolipid protein (PLP) is a key element in the mechanism of myelin assembly. Upon biosynthesis, PLP traffics to myelin membranes via syntaxin 3-mediated docking at the apical-surface-like cell body plasma membrane, which is followed by subsequent internalization and transport to the basolateral-surface-like myelin sheet. Pulse-chase experiments, in conjunction with surface biotinylation and organelle fractionation, reveal that following biosynthesis, PLP is transported to the cell body surface in Triton X-100 (TX-100)-resistant microdomains. At the plasma membrane, PLP transiently resides within these microdomains and its lateral dissipation is followed by segregation into 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-resistant domains, internalization, and subsequent transport toward the myelin membrane. Sulfatide triggers PLP's reallocation from TX-100- into CHAPS-resistant membrane domains, while inhibition of sulfatide biosynthesis inhibits transcytotic PLP transport. Taking these findings together, we propose a model in which PLP transport to the myelin membrane proceeds via a transcytotic mechanism mediated by sulfatide and characterized by a conformational alteration and dynamic, i.e., transient, partitioning of PLP into distinct membrane microdomains involved in biosynthetic and transcytotic transport.

3.2287 Outer-Inner Membrane Vesicles Naturally Secreted by Gram-Negative Pathogenic Bacteria
Pérez-Cruz C, Delgado L, López-Iglesias C, Mercade E
*PloS One, **10(1)**, e0116896 (2015)*

Outer-inner membrane vesicles (O-IMVs) were recently described as a new type of membrane vesicle secreted by the Antarctic bacterium ***Shewanella vesiculosa*** M7T. Their formation is characterized by the protrusion of both outer and plasma membranes, which pulls cytoplasmic components into the vesicles. To demonstrate that this is not a singular phenomenon in a bacterium occurring in an extreme environment, the identification of O-IMVs in pathogenic bacteria was undertaken. With this aim, a structural study by Transmission Electron Microscopy (TEM) and Cryo-transmission electron microscopy (Cryo-TEM) was carried out, confirming that O-IMVs are also secreted by Gram-negative pathogenic bacteria such as ***Neisseria gonorrhoeae***, ***Pseudomonas aeruginosa*** PAO1 and ***Acinetobacter baumannii*** AB41, in which they represent between 0.23% and 1.2% of total vesicles produced. DNA and ATP, which are components solely found in the cell cytoplasm, were identified within membrane vesicles of these strains. The presence of DNA inside the O-IMVs produced by ***N. gonorrhoeae*** was confirmed by gold DNA immunolabeling with a specific monoclonal IgM against double-stranded DNA. A proteomic analysis of ***N. gonorrhoeae***-derived membrane vesicles identified proteins from the cytoplasm and plasma membrane. This confirmation of O-IMV extends the hitherto uniform definition of membrane vesicles in Gram-negative bacteria and explains the presence of components in membrane vesicles such as DNA, cytoplasmic and inner membrane proteins, as well as ATP, detected for the first time. The production of these O-IMVs by pathogenic Gram-negative bacteria opens up new areas of study related to their involvement in lateral gene transfer, the transfer of cytoplasmic proteins, as well as the functionality and role of ATP detected in these new vesicles.

3.2288 Mammalian ER mannosidase I resides in quality control vesicles, where it encounters its glycoprotein substrates
Benyair, R., Ogen-Shtern, N., Mazkereth, N., Shai, B., Ehrlich, M and Lederkremer, G.Z.
*Mol. Biol. Cell, **26**, 172-184 (2015)*

Endoplasmic reticulum α1,2 mannosidase I (ERManI), a central component of ER quality control and ER-associated degradation (ERAD), acts as a timer enzyme, modifying N-linked sugar chains of glycoproteins with time. This process halts glycoprotein folding attempts when necessary and targets terminally

misfolded glycoproteins to ERAD. Despite the importance of ERManI in maintenance of glycoprotein quality control, fundamental questions regarding this enzyme remain controversial. One such question is the subcellular localization of ERManI, which has been suggested to localize to the ER membrane, the ER-derived quality control compartment (ERQC), and, surprisingly, recently to the Golgi apparatus. To try to clarify this controversy, we applied a series of approaches that indicate that ERManI is located, at the steady state, in quality control vesicles (QCVs) to which ERAD substrates are transported and in which they interact with the enzyme. Both endogenous and exogenously expressed ERManI migrate at an ER-like density on iodixanol gradients, suggesting that the QCVs are derived from the ER. The QCVs are highly mobile, displaying dynamics that are dependent on microtubules and COP-II but not on COP-I vesicle machinery. Under ER stress conditions, the QCVs converge in a juxtanuclear region, at the ERQC, as previously reported. Our results also suggest that ERManI is turned over by an active autophagic process. Of importance, we found that membrane disturbance, as is common in immunofluorescence methods, leads to an artificial appearance of ERManI in a Golgi pattern.

3.2289 **Characterization of VAMP isoforms in 3T3-L1 adipocytes: implications for GLUT4 trafficking**
Sadler, J.B., Bryant, N.J. and Gould, G.W.
*Mol. Biol. Cell, **26**, 530-536 (2015)*

The fusion of GLUT4-containing vesicles with the plasma membrane of adipocytes is a key facet of insulin action. This process is mediated by the formation of functional soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complexes between the plasma membrane t-SNARE complex and the vesicle v-SNARE or VAMP. The t-SNARE complex consists of Syntaxin4 and SNAP23, and whereas many studies identify VAMP2 as the v-SNARE, others suggest that either VAMP3 or VAMP8 may also fulfil this role. Here we characterized the levels of expression, distribution, and association of all the VAMPs expressed in 3T3-L1 adipocytes to provide the first systematic analysis of all members of this protein family for any cell type. Despite our finding that all VAMP isoforms form SDS-resistant SNARE complexes with Syntaxin4/SNAP23 in vitro, a combination of levels of expression (which vary by >30-fold), subcellular distribution, and coimmunoprecipitation analyses lead us to propose that VAMP2 is the major v-SNARE involved in GLUT4 trafficking to the surface of 3T3-L1 adipocytes.

3.2290 **Cell Type-Specific Affinity Purification of Nuclei for Chromatin Profiling in Whole Animals**
Steiner, F.A. and Henikoff, S.
*Methods in Mol. Biol., **1228**, 3-14 (2015)*

Analyzing cell differentiation during development in a complex organism requires the analysis of expression and chromatin profiles in individual cell types. Our laboratory has developed a simple and generally applicable strategy to purify specific cell types from whole organisms for simultaneous analysis of chromatin and expression. The method, termed INTACT for Isolation of Nuclei TAgged in specific Cell Types, depends on the expression of an affinity-tagged nuclear envelope protein in the cell type of interest. These nuclei can be affinity-purified from the total pool of nuclei and used as a source for RNA and chromatin. The method serves as a simple and scalable alternative to FACS sorting or laser capture microscopy to circumvent the need for expensive equipment and specialized skills. This chapter provides detailed protocols for the cell-type specific purification of nuclei from *Caenorhabditis elegans*.

3.2291 **Transferrin receptor expression in serum exosomes as a marker of regenerative anaemia in the horse**
Rout, E.D., Webb, T.L., Laurence, H.M., Long, L. and Olver, C.S.
*Equine Veterinary Journal, **47(1)**, 101-106 (2015)*

Reasons for performing study
Evaluation of erythrocyte regeneration in horses is challenging, as they do not release reticulocytes into the peripheral blood. This study investigated transferrin receptor 1 (TfR1) expression in exosomes as a noninvasive method of characterising the regenerative response in anaemic horses.
Objectives
To quantify TfR1 in ultraprecipitate of serum in horses before and after phlebotomy-induced anaemia, and to identify exosomes as the source of TfR1. The hypothesis was that serum exosomal TfR1 expression would increase during a regenerative response.
Study design
Experimental model of anaemia.
Methods

Six horses were phlebotomised to achieve a 25% decrease in packed cell volume. Transferrin receptor 1 quantity in exosomes was determined by western blot and relative densitometry before and after phlebotomy. The size and density of the TfR1-associated particles were confirmed by transmission electron microscopy and density gradient centrifugation, respectively.

Results

Regenerative anaemia was confirmed by decreased packed cell volumes and decreased myeloid:erythroid ratios in the bone marrow. In all 6 horses, TfR1 expression increased between Days 7 and 10. Mean TfR1 levels peaked on Day 10 and at 3-fold higher than levels on Day 0. Appropriately sized particles were evident on transmission electron microscopy and sucrose density gradient fractions expected to contain exosomes also contained TfR1.

Conclusions

These data indicate that TfR1 expression in serum exosomes may provide a marker for regeneration in anaemic horses.

3.2292 Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling

Plociennikowska, A., hromada-Judycka, A., Borzecka, K. and Kwiatkowska, K.
*Cell. Mol. Life Sci., **72**, 557-581 (2015)*

Toll-like receptor 4 (TLR4) is activated by lipopolysaccharide (LPS), a component of Gram-negative bacteria to induce production of pro-inflammatory mediators aiming at eradication of the bacteria. Dysregulation of the host responses to LPS can lead to a systemic inflammatory condition named sepsis. In a typical scenario, activation of TLR4 is preceded by binding of LPS to CD14 protein anchored in cholesterol- and sphingolipid-rich microdomains of the plasma membrane called rafts. CD14 then transfers the LPS to the TLR4/MD-2 complex which dimerizes and triggers MyD88- and TRIF-dependent production of pro-inflammatory cytokines and type I interferons. The TRIF-dependent signaling is linked with endocytosis of the activated TLR4, which is controlled by CD14. In addition to CD14, other raft proteins like Lyn tyrosine kinase of the Src family, acid sphingomyelinase, CD44, Hsp70, and CD36 participate in the TLR4 signaling triggered by LPS and non-microbial endogenous ligands. In this review, we summarize the current state of the knowledge on the involvement of rafts in TLR4 signaling, with an emphasis on how the raft proteins regulate the TLR4 signaling pathways. CD14-bearing rafts, and possibly CD36-rich rafts, are believed to be preferred sites of the assembly of a multimolecular complex which mediates the endocytosis of activated TLR4.

3.2293 Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing

Lane, R.E., Korbie, D., Anderson, W., Vaidyanathan, R. and Trau, M.
*Scientific Reports, **5**:7639 (2015)*

Exosomes are vesicles which have garnered interest due to their diagnostic and therapeutic potential. Isolation of pure yields of exosomes from complex biological fluids whilst preserving their physical characteristics is critical for downstream applications. In this study, we use 100 nm-liposomes from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol as a model system as a model system to assess the effect of exosome isolation protocols on vesicle recovery and size distribution using a single-particle analysis method. We demonstrate that liposome size distribution and ζ-potential are comparable to extracted exosomes, making them an ideal model for comparison studies. Four different purification protocols were evaluated, with liposomes robustly isolated by three of them. Recovered yields varied and liposome size distribution was unaltered during processing, suggesting that these protocols do not induce particle aggregation. This leads us to conclude that the size distribution profile and characteristics of vesicles are stably maintained during processing and purification, suggesting that reports detailing how exosomes derived from tumour cells differ in size to those from normal cells are reporting a real phenomenon. However, we hypothesize that larger particles present in most purified exosome samples represent co-purified contaminating non-exosome debris. These isolation techniques are therefore likely nonspecific and may co-isolate non-exosome material of similar physical properties.

3.2294 DRAM1 regulates apoptosis through increasing protein levels and lysosomal localization of BAX

Guan, J.J., Zhang, X.D., Sun, W., Qi, L., Wu, J.C. and Qin, Z.H.
*Cell Death and Disease, **6**, e1624 (2015)*

DRAM1 (DNA damage-regulated autophagy modulator 1) is a TP53 target gene that modulates autophagy and apoptosis. We previously found that DRAM1 increased autophagy flux by promoting lysosomal

acidification and protease activation. However, the molecular mechanisms by which DRAM1 regulates apoptosis are not clearly defined. Here we report a novel pathway by which DRAM1 regulates apoptosis involving BAX and lysosomes. A549 or HeLa cells were treated with the mitochondrial complex II inhibitor, 3-nitropropionic acid (3NP), or an anticancer drug, doxorubicin. Changes in the protein and mRNA levels of BAX and DRAM1 and the role of DRAM1 in BAX induction were determined. The interaction between DRAM1 and BAX and its effect on BAX degradation, BAX lysosomal localization, the release of cathepsin B and cytochrome c by BAX and the role of BAX in 3NP- or doxorubicin-induced cell death were studied. The results showed that BAX, a proapoptotic protein, was induced by DRAM1 in a transcription-independent manner. BAX was degraded by autophagy under basal conditions; however, its degradation was inhibited when DRAM1 expression was induced. There was a protein interaction between DRAM1 and BAX and this interaction prolonged the half-life of BAX. Furthermore, upregulated DRAM1 recruited BAX to lysosomes, leading to the release of lysosomal cathepsin B and cleavage of BID (BH3-interacting domain death agonist). BAX mediated the release of mitochondrial cytochrome c, activation of caspase-3 and cell death partially through the lysosome-cathepsin B-tBid pathway. These results indicate that DRAM1 regulates apoptosis by inhibiting BAX degradation. In addition to mitochondria, lysosomes may also be involved in BAX-initiated apoptosis.

3.2295 **An oncogenic role of Agrin in regulating focal adhesion integrity in hepatocellular carcinoma**
Chakraborty, S., Lakshmanan, M., Swa, H.L.F., Chen, J., Zhang, X., Ong, Y.S., Loo, L.S., Akincilar, S.C., Gunaratne, J., tergaonkar, V., Hui, K.M. and Hong, W.
*Nature Communications, **6**:6184 (2015)*

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths globally. The identity and role of cell surface molecules driving complex biological events leading to HCC progression are poorly understood, hence representing major lacunae in HCC therapies. Here, combining SILAC quantitative proteomics and biochemical approaches, we uncover a critical oncogenic role of Agrin, which is overexpressed and secreted in HCC. Agrin enhances cellular proliferation, migration and oncogenic signalling. Mechanistically, Agrin's extracellular matrix sensor activity provides oncogenic cues to regulate Arp2/3-dependent ruffling, invadopodia formation and epithelial–mesenchymal transition through sustained focal adhesion integrity that drives liver tumorigenesis. Furthermore, Agrin signalling through Lrp4-muscle-specific tyrosine kinase (MuSK) forms a critical oncogenic axis. Importantly, antibodies targeting Agrin reduced oncogenic signalling and tumour growth *in vivo*. Together, we demonstrate that Agrin is frequently upregulated and important for oncogenic property of HCC, and is an attractive target for antibody therapy.

3.2296 **Proteomic Analysis of Isolated Ciliary Transition Zones Reveals the Presence of ESCRT Proteins**
Diener, D.R., Lupetti, P. and Rosenbaum, J.L.
*Current Biology, **25(3)**, 379-384 (2015)*

The transition zone (TZ) is a specialized region of the cilium characterized by Y-shaped connectors between the microtubules of the ciliary axoneme and the ciliary membrane [1]. Located near the base of the cilium, the TZ is in the prime location to act as a gate for proteins into and out of the ciliary compartment, a role supported by experimental evidence [2–6]. The importance of the TZ has been underscored by studies showing that mutations affecting proteins located in the TZ result in cilia-related diseases, or ciliopathies, presenting symptoms including renal cysts, retinal degeneration, and situs inversus [7–9]. Some TZ proteins have been identified and shown to interact with each other through coprecipitation studies in vertebrate cells [4, 10, 11] and genetics studies in *C. elegans* [3]. As a distinct approach to identify TZ proteins, we have taken advantage of the biology of *Chlamydomonas* to isolate TZs. Proteomic analysis identified 115 proteins, ten of which were known TZ proteins related to ciliopathies, indicating that the preparation was highly enriched for TZs. Interestingly, six proteins of the endosomal sorting complexes required for transport (ESCRT) were also associated with the TZs. Identification of these and other proteins in the TZ will provide new insights into functions of the TZ, as well as candidate ciliopathy genes.

3.2297 **A Protocol for Exosome Isolation and Characterization: Evaluation of Ultracentrifugation, Density-Gradient Separation, and Immunoaffinity Capture Methods**
Greening, D.W., Xu, R., Ji, H., Tauro, B.J. and Simpson, R.J.
Methods in Mol. Biol., 1295, 179-209 (2015)

Exosomes are 40–150 nm extracellular vesicles that are released from a multitude of cell types, and perform diverse cellular functions including intercellular communication, antigen presentation, and

transfer of tumorigenic proteins, mRNA and miRNA. Exosomes are important regulators of the cellular niche, and their altered characteristics in many diseases, such as cancer, suggest their importance for diagnostic and therapeutic applications, and as drug delivery vehicles. Exosomes have been purified from biological fluids and in vitro cell cultures using a variety of strategies and techniques. In this chapter, we reveal the protocol and key insights into the isolation, purification and characterization of exosomes, distinct from shed microvesicles and apoptotic blebs. Using the colorectal cancer cell line LIM1863 as a cell model, a comprehensive evaluation of exosome isolation methods including ultracentrifugation (UC-Exos), OptiPrep™ density-based separation (DG-Exos), and immunoaffinity capture using anti-EpCAM-coated magnetic beads (IAC-Exos) were examined. All exosome isolation methodologies contained 40–150 nm vesicles based on electron microscopy, and positive for exosome markers (Alix, TSG101, HSP70) based on immunoblotting. This protocol employed a proteomic profiling approach to characterize the protein composition of exosomes, and label-free spectral counting to evaluate the effectiveness of each method in exosome isolation. Based on the number of MS/MS spectra identified for exosome markers and proteins associated with their biogenesis, trafficking, and release, IAC-Exos was shown to be the most effective method to isolate exosomes. However, the use of density-based separation (DG-Exos) provides significant advantages for exosome isolation when the use of immunoaffinity capture is limited (due to antibody availability and suitability of exosome markers).

3.2298 UBC9-dependent Association between Calnexin and Protein Tyrosine Phosphatase 1B (PTP1B) at the Endoplasmic Reticulum

Lee, D., Kraus, A., Prins, D., Groenendyk, J., Aubry, I., Liu, W-X., Li, H-D., Julien, O., Touret, N., Sykes, B.D., Tremblay, M.L. and Michalak, M.
J. Biol. Chem., ***290(9)***, *5725-5738 (2015)*

Calnexin is a type I integral endoplasmic reticulum (ER) membrane protein, molecular chaperone, and a component of the translocon. We discovered a novel interaction between the calnexin cytoplasmic domain and UBC9, a SUMOylation E2 ligase, which modified the calnexin cytoplasmic domain by the addition of SUMO. We demonstrated that calnexin interaction with the SUMOylation machinery modulates an interaction with protein tyrosine phosphatase 1B (PTP1B), an ER-associated protein tyrosine phosphatase involved in the negative regulation of insulin and leptin signaling. We showed that calnexin and PTP1B form UBC9-dependent complexes, revealing a previously unrecognized contribution of calnexin to the retention of PTP1B at the ER membrane. This work shows that the SUMOylation machinery links two ER proteins from divergent pathways to potentially affect cellular protein quality control and energy metabolism.

3.2299 Lipid-induced NOX2 activation inhibits autophagic flux by impairing lysosomal enzyme activity

Jaishy, B., Zhang, Q., Chung, H.S., Riehle, C., Soto, J., Jenkins, S., Abel, P., Cowart, L.A., Van Eyk, J.E. and Abel, E.D.
J. Lipid Res., ***56***, *546-561 (2015)*

Autophagy is a catabolic process involved in maintaining energy and organelle homeostasis. The relationship between obesity and the regulation of autophagy is cell type specific. Despite adverse consequences of obesity on cardiac structure and function, the contribution of altered cardiac autophagy in response to fatty acid overload is incompletely understood. Here, we report the suppression of autophagosome clearance and the activation of NADPH oxidase (Nox)2 in both high fat-fed murine hearts and palmitate-treated H9C2 cardiomyocytes (CMs). Defective autophagosome clearance is secondary to superoxide-dependent impairment of lysosomal acidification and enzyme activity in palmitate-treated CMs. Inhibition of Nox2 prevented superoxide overproduction, restored lysosome acidification and enzyme activity, and reduced autophagosome accumulation in palmitate-treated CMs. Palmitate-induced Nox2 activation was dependent on the activation of classical protein kinase Cs (PKCs), specifically PKCβII. These findings reveal a novel mechanism linking lipotoxicity with a PKCβ-Nox2-mediated impairment in pH-dependent lysosomal enzyme activity that diminishes autophagic turnover in CMs.

3.2300 ABCB4 exports phosphatidylcholine in a sphingomyelin-dependent manner

Zhao, Y., Ishigami, M., Nagao, K., hanada, K., Kono, N., Arai, H., matssuo, M., Kioka, N. and Ueda, K.
J. Lipid Res., ***56***, *644-652 (2015)*

ABCB4, which is specifically expressed on the canalicular membrane of hepatocytes, exports phosphatidylcholine (PC) into bile. Because SM depletion increases cellular PC content and stimulates PC

and cholesterol efflux by ABCA1, a key transporter involved in generation of HDL, we predicted that SM depletion also stimulates PC efflux through ABCB4. To test this prediction, we compared the lipid efflux activity of ABCB4 and ABCA1 under SM depletion induced by two different types of inhibitors for SM synthesis, myriocin and (*1R*,*3S*)-*N*-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide, in human embryonic kidney 293 and baby hamster kidney cells. Unexpectedly, SM depletion exerted opposite effects on ABCB4 and ABCA1, suppressing PC efflux through ABCB4 while stimulating efflux through ABCA1. Both ABCB4 and ABCA1 were recovered from Triton-X-100-soluble membranes, but ABCB4 was mainly recovered from CHAPS-insoluble SM-rich membranes, whereas ABCA1 was recovered from CHAPS-soluble membranes. These results suggest that a SM-rich membrane environment is required for ABCB4 to function. ABCB4 must have evolved to exert its maximum activity in the SM-rich membrane environment of the canalicular membrane, where it transports PC as the physiological substrate.

3.2301 Identification of a Family of Fatty-Acid-Speciated Sonic Hedgehog Proteins, Whose Members Display Differential Biological Properties
Long, J., Tokhunts, R., Ahn, N.G. and Robbins, D.J:
*Cell Reports, **10**, 1280-1287 (2015)*

Hedgehog (HH) proteins are proteolytically processed into a biologically active form that is covalently modified by cholesterol and palmitate. However, most studies of HH biogenesis have characterized protein from cells in which HH is overexpressed. We purified Sonic Hedgehog (SHH) from cells expressing physiologically relevant levels and showed that it was more potent than SHH isolated from overexpressing cells. Furthermore, the SHH in our preparations was modified with a diverse spectrum of fatty acids on its amino termini, and this spectrum of fatty acids varied dramatically depending on the growth conditions of the cells. The fatty acid composition of SHH affected its trafficking to lipid rafts as well as its potency. Our results suggest that HH proteins exist as a family of diverse lipid-speciated proteins that might be altered in different physiological and pathological contexts in order to regulate distinct properties of HH proteins.

3.2302 Subcellular localization and activation of ADAM proteases in the context of FasL shedding in T lymphocytes
Ebsen, H., Lettau, M:, kabelitz, D. and Janssen, O.
*Mol. Immunol., **65**, 416-428 (2015)*

The “A Disintegrin And Metalloproteinases” (ADAMs) form a subgroup of the metzincin endopeptidases. Proteolytically active members of this protein family act as sheddases and govern key processes in development and inflammation by regulating cell surface expression and release of cytokines, growth factors, adhesion molecules and their receptors. In T lymphocytes, ADAM10 sheds the death factor Fas Ligand (FasL) and thereby regulates T cell activation, death and effector function. Although FasL shedding by ADAM10 was confirmed in several studies, its regulation is still poorly defined. We recently reported that ADAM10 is highly abundant on T cells whereas its close relative ADAM17 is expressed at low levels and transiently appears at the cell surface upon stimulation. Since FasL is also stored intracellularly and brought to the plasma membrane upon stimulation, we addressed where the death factor gets exposed to ADAM proteases. We report for the first time that both ADAM10 and ADAM17 are associated with FasL-containing secretory lysosomes. Moreover, we demonstrate that TCR/CD3/CD28-stimulation induces a partial positioning of both proteases and FasL to lipid rafts and only the activation-induced raft-positioning results in FasL processing. TCR/CD3/CD28-induced FasL proteolysis is markedly affected by reducing both ADAM10 and ADAM17 protein levels, indicating that in human T cells also ADAM17 is implicated in FasL processing. Since FasL shedding is affected by cholesterol depletion and by inhibition of Src kinases or palmitoylation, we conclude that it requires mobilization and co-positioning of ADAM proteases in lipid raft-like platforms associated with an activation of raft-associated Src-family kinases.

3.2303 Host ESCRT Proteins Are Required for Bromovirus RNA Replication Compartment Assembly and Function
Diaz, A., Zhang, J., Ollwerther, A., Wang, X. and Ahlquist, P.
*PloS Pathogens, **11(3)**, e1004742 (2015)*

Positive-strand RNA viruses genome replication invariably is associated with vesicles or other rearranged cellular membranes. Brome mosaic virus (BMV) RNA replication occurs on perinuclear endoplasmic reticulum (ER) membranes in ~70 nm vesicular invaginations (spherules). BMV RNA replication vesicles show multiple parallels with membrane-enveloped, budding retrovirus virions, whose envelopment and

release depend on the host ESCRT (endosomal sorting complexes required for transport) membrane-remodeling machinery. We now find that deleting components of the ESCRT pathway results in at least two distinct BMV phenotypes. One group of genes regulate RNA replication and the frequency of viral replication complex formation, but had no effect on spherule size, while a second group of genes regulate RNA replication in a way or ways independent of spherule formation. In particular, deleting *SNF7* inhibits BMV RNA replication > 25-fold and abolishes detectable BMV spherule formation, even though the BMV RNA replication proteins accumulate and localize normally on perinuclear ER membranes. Moreover, BMV ESCRT recruitment and spherule assembly depend on different sets of protein-protein interactions from those used by multivesicular body vesicles, HIV-1 virion budding, or tomato bushy stunt virus (TBSV) spherule formation. These and other data demonstrate that BMV requires cellular ESCRT components for proper formation and function of its vesicular RNA replication compartments. The results highlight growing but diverse interactions of ESCRT factors with many viruses and viral processes, and potential value of the ESCRT pathway as a target for broad-spectrum antiviral resistance.

3.2304 Polysome arrest restricts miRNA turnover by preventing exosomal export of miRNA in growth-retarded mammalian cells

Ghosh, S., Bose, M., Ray, A. and Bhattacharyya, S.N.
*Mol. Biol. Cell, **26**, 1072-1083 (2015)*

MicroRNAs (miRNAs) are tiny posttranscriptional regulators of gene expression in metazoan cells, where activity and abundance of miRNAs are tightly controlled. Regulated turnover of these regulatory RNAs is important to optimize cellular response to external stimuli. We report that the stability of mature miRNAs increases inversely with cell proliferation, and the increased number of microribonucleoproteins (miRNPs) in growth-restricted mammalian cells are in turn associated with polysomes. This heightened association of miRNA with polysomes also elicits reduced degradation of target mRNAs and impaired extracellular export of miRNA via exosomes. Overall polysome sequestration contributes to an increase of cellular miRNA levels but without an increase in miRNA activity. Therefore miRNA activity and turnover can be controlled by subcellular distribution of miRNPs that may get differentially regulated as a function of cell growth in mammalian cells.

3.2305 The Assembly of GM1 Glycolipid- and Cholesterol-Enriched Raft-Like Membrane Microdomains Is Important for Giardial Encystation

De Chatterjee, A., Mendez, T.L., Roychowdury, S. and Das, S.
Infect. Immun., 83(5), 2030-2042 (2015)

Although encystation (or cyst formation) is an important step of the life cycle of Giardia, the cellular events that trigger encystation are poorly understood. Because membrane microdomains are involved in inducing growth and differentiation in many eukaryotes, we wondered if these raft-like domains are assembled by this parasite and participate in the encystation process. Since the GM1 ganglioside is a major constituent of mammalian lipid rafts (LRs) and known to react with cholera toxin B (CTXB), we used Alexa Fluor-conjugated CTXB and GM1 antibodies to detect giardial LRs. Raft-like structures in trophozoites are located in the plasma membranes and on the periphery of ventral discs. In cysts, however, they are localized in the membranes beneath the cyst wall. Nystatin and filipin III, two cholesterol-binding agents, and oseltamivir (Tamiflu), a viral neuraminidase inhibitor, disassembled the microdomains, as evidenced by reduced staining of trophozoites with CTXB and GM1 antibodies. GM1- and cholesterol-enriched LRs were isolated from Giardia by density gradient centrifugation and found to be sensitive to nystatin and oseltamivir. The involvement of LRs in encystation could be supported by the observation that raft inhibitors interrupted the biogenesis of encystation-specific vesicles and cyst production. Furthermore, culturing of trophozoites in dialyzed medium containing fetal bovine serum (which is low in cholesterol) reduced raft assembly and encystation, which could be rescued by adding cholesterol from the outside. Our results suggest that Giardia is able to form GM1- and cholesterol-enriched lipid rafts and these raft domains are important for encystation.

3.2306 Lipidomic and proteomic characterization of platelet extracellular vesicle subfractions from senescent platelets

Pienimaeki-Roemer, A., Kuhlmann, K., Böttcher, A., Konovalova, T., Black, A., Orso, E., Liebish, G., Ahrens, M., Eisenacher, M., Meyer, H.E. and Schmitz, G.
*Transfusion, **55(3)**, 507-521 (2015)*

Background

Platelets (PLTs) in stored PLT concentrates (PLCs) release PLT extracellular vesicles (PL-EVs) induced by senescence and activation, resembling the PLT storage lesion. No comprehensive classification or molecular characterization of senescence-induced PL-EVs exists to understand PL-EV heterogeneity.
Study Design and Methods
PL-EVs from 5-day-stored PLCs from healthy individuals were isolated and subfractionated by differential centrifugation, filtration, and density gradient ultracentrifugation into five PLT microvesicle (PL-MV) subfractions (Fraction [F]1-F5) and PLT exosomes (PL-EXs). PL-EV size, concentration, and composition were analyzed by nanoparticle tracking analysis, flow cytometry, and lipid and protein mass spectrometry. Protein data were verified by Western blot.
Results
PL-EVs showed overlapping mean particle sizes of 180 to 260 nm, but differed significantly in composition. Less dense, intermediate, and dense PL-MVs enriched specific lipidomic and proteomic markers related to the plasma membrane, intracellular membranes, PLT granules, mitochondria, and PLT activation. α-Synuclein (81% of total) accumulated in F1 and F2, amyloid-β (Aβ) precursor protein in F3 and F4 (84%), and apolipoprotein (Apo)E (88%) and ApoJ (92%) in F3 to F5. PL-EXs enriched lipid species and proteins, with high abundance of lipid raft, PLT adhesion, and immune response–related markers.
Conclusion
Differential lipid and protein compositions of PL-EVs suggest their unique cellular origins and functions, partly overlapping with PLT granule secretion. Dense PL-MVs might represent autophagic vesicles released during PLT activation and apoptosis and PL-EXs resemble lipid rafts, with a potential role in PLT aggregation and immunity. Segregation of α-synuclein and Aβ precursor protein, ApoE, and ApoJ into less dense and dense PL-MVs, respectively, show their differential carrier role of neurologic disease–related cargo.

3.2307 Phospholipids of tumor extracellular vesicles stratify gefitinib-resistant nonsmall cell lung cancer cells from gefitinib-sensitive cells
Jung, J.H., Lee, M.Y., Choi, D-Y., Lee, J.W., You, S., Lee, K.Y., Kim, J. and Kim, K.P.
*Proteomics, **15(4)**, 824-835 (2015)*

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as gefitinib are one of gold standard treatment options for nonsmall-cell lung cancer (NSCLC) patients, which eventually fail due to the acquired resistance and relapse because of the development of secondary activating mutations such as T790M in EGFR. Predicting chemo-responsiveness of cancer patients provides a major challenge in chemotherapy. The goal of the present study is to determine whether phospholipid signatures of tumor extracellular vesicles (EV) are associated with gefitinib-resistance of NSCLC. A sophisticated MS-based shotgun lipidomic assays were performed for in-depth analysis of the lipidomes of gefitinib-resistant (PC9R) and responsive (PC9) NSCLC cells and their shed EV from these cell lines (PC9EV or PC9REV). Lipid MALDI-MS analysis showed that EV phospholipid composition was significantly distinct in PC9R, compared to PC9 cells. Following statistical analyses has identified 35 (20 positive and 15 negative ion mode) differentially regulated lipids, which are significantly over- or underexpressed in PC9R EV, compared to PC9 EV (p value < 0.01, fold change > 1.5). Our phospholipid signatures suggest that EV associates with drug sensitivity, which is worthy of additional investigation to assess chemoresistance in patients with NSCLC treated with anti-EGFR TKIs.

3.2308 Evidence for the functioning of a Cl−/H+ antiporter in the membranes isolated from root cells of the halophyte Suaeda altissima and enriched with Golgi membranes
Shuvalov, A.V., Orlava, J.V., Khalilova, L.A., Myasoedov, N.A., Andreev, I.M., Belyaev, D.V. and Balkonin, Y.V.
*Russian J. Plant Physiol., **61(1)**, 45-56 (2015)*

Cl^-/H^+ exchange activity in the membranes isolated from the root cells of the halophyte *Suaeda altissima* (L.) Pall. was originally revealed and characterized. The membrane vesicles were isolated by centrifugation of microsomes in a continuous iodixanol density gradient. The highest activity of latent inosine phosphatase, a marker of Golgi membranes, was localized in the upper part of the gradient, indicating its enrichment with Golgi membranes. The same part of the gradient was characterized by the highest Cl^-/H^+ exchange rate. The Cl^-/H^+ exchange activity was detected as electrogenic ΔpCl-dependent H^+ transport monitored as changes in differential absorbance of a ΔpH-probe acridine orange, or as changes in fluorescence excitation spectrum of a pH-probe pyranine loaded into the vesicles. Generation of transmembrane electric potential (Δψ) during the Cl^-/H^+ exchange was assayed as changes in differential

absorbance of a Δψ-probe safranin O. Establishing the transmembrane ΔpCl inward vesicles resulted in H^+ efflux sensitive to DIDS (4,4′-diisothiocyano-2,2′-stylbene-disulfonic acid), an inhibitor of chloride transporters and channels, and generation of Δψ negative inside. To maintain the ΔpCl-dependent H^+ efflux from the vesicles, either the presence of a penetrating cation tetraphenylphosphonium neutralizing negative charges inside the vesicles or null K^+ diffusion potential across the membranes was required. The results demonstrate the activity of an electrogenic Cl^-/H^+ antiporter in the fraction enriched with Golgi membranes. We hypothesize that the Cl^-/H^+ antiporter is involved into the regulation of cytoplasmic Cl^- concentrations by vesicular trafficking of Cl^- from the cytoplasm to the vacuole by endosomes, derivatives of Golgi membranes.

3.2309 Detergent-Resistant Membrane Association of NS2 and E2 during Hepatitis C Virus Replication
Shanmugam, S., Saravanabalaji, D. and Yi, M.
J. Virol., ***89(8)***, *4562-4574 (2015)*

Previously, we demonstrated that the efficiency of hepatitis C virus (HCV) E2-p7 processing regulates p7-dependent NS2 localization to putative virus assembly sites near lipid droplets (LD). In this study, we have employed subcellular fractionations and membrane flotation assays to demonstrate that NS2 associates with detergent-resistant membranes (DRM) in a p7-dependent manner. However, p7 likely plays an indirect role in this process, since only the background level of p7 was detectable in the DRM fractions. Our data also suggest that the p7-NS2 precursor is not involved in NS2 recruitment to the DRM, despite its apparent targeting to this location. Deletion of NS2 specifically inhibited E2 localization to the DRM, indicating that NS2 regulates this process. Treatment of cells with methyl-β-cyclodextrin (MβCD) significantly reduced the DRM association of Core, NS2, and E2 and reduced infectious HCV production. Since disruption of the DRM localization of NS2 and E2, either due to p7 and NS2 defects, respectively, or by MβCD treatment, inhibited infectious HCV production, these proteins' associations with the DRM likely play an important role during HCV assembly. Interestingly, we detected the HCV replication-dependent accumulation of ApoE in the DRM fractions. Taking into consideration the facts that ApoE was shown to be a major determinant for infectious HCV particle production at the postenvelopment step and that the HCV Core protein strongly associates with the DRM, recruitment of E2 and ApoE to the DRM may allow the efficient coordination of Core particle envelopment and postenvelopment events at the DRM to generate infectious HCV production.

3.2310 Human T-Cell Leukemia Virus Type 1 (HTLV-1) Tax Requires CADM1/TSLC1 for Inactivation of the NF-κB Inhibitor A20 and Constitutive NF-κB Signaling
Pujari, R., Hunte, R., Thomas, R., van der Weyden, L., Rauch, D., Ratner, L., Nyborg, J.K., Ramos, J.C., Takai, Y. and Shembade, N.
PloS Pathogens, ***11(3)***, *e1004721 (2015)*

Persistent activation of NF-κB by the Human T-cell leukemia virus type 1 (HTLV-1) oncoprotein, Tax, is vital for the development and pathogenesis of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). K63-linked polyubiquitinated Tax activates the IKK complex in the plasma membrane-associated lipid raft microdomain. Tax also interacts with TAX1BP1 to inactivate the NF-κB negative regulatory ubiquitin-editing A20 enzyme complex. However, the molecular mechanisms of Tax-mediated IKK activation and A20 protein complex inactivation are poorly understood. Here, we demonstrated that membrane associated CADM1 (Cell adhesion molecule1) recruits Ubc13 to Tax, causing K63-linked polyubiquitination of Tax, and IKK complex activation in the membrane lipid raft. The c-terminal cytoplasmic tail containing PDZ binding motif of CADM1 is critical for Tax to maintain persistent NF-κB activation. Finally, Tax failed to inactivate the NF-κB negative regulator ubiquitin-editing enzyme A20 complex, and activate the IKK complex in the lipid raft in absence of CADM1. Our results thus indicate that CADM1 functions as a critical scaffold molecule for Tax and Ubc13 to form a cellular complex with NEMO, TAX1BP1 and NRP, to activate the IKK complex in the plasma membrane-associated lipid rafts, to inactivate NF-κB negative regulators, and maintain persistent NF-κB activation in HTLV-1 infected cells.

3.2311 Dietary and biliary phosphatidylcholine activates PKC{zeta} in rat intestine
Siddiqi, S. and Mansbach II, C.M.
J. Lipid Res., ***56***, *859-870 (2015)*

Chylomicron output by the intestine is proportional to intestinal phosphatidylcholine (PC) delivery. Using five different variations of PC delivery to the intestine, we found that lyso-phosphatidylcholine (lyso-PC),

the absorbed form of PC, concentrations in the cytosol (0 to 0.45 nM) were proportional to the input rate. The activity of protein kinase C (PKC)ζ, which controls prechylomicron output rate by the endoplasmic reticulum (ER), correlated with the lyso-PC concentration suggesting that it may be a PKCζ activator. Using recombinant PKCζ, the K_m for lyso-PC activation was 1.49 nM and the V_{max} 1.12 nM, more than the maximal lyso-PC concentration in cytosol, 0.45 nM. Among the phospholipids and their lyso derivatives, lyso-PC was the most potent activator of PKCζ and the only one whose cytosolic concentration suggested that it could be a physiological activator because other phospholipid concentrations were negligible. PKCζ was on the surface of the dietary fatty acid transport vesicle, the caveolin-1-containing endocytic vesicle. Once activated, PKCζ, eluted off the vesicle. A conformational change in PKCζ on activation was suggested by limited proteolysis. We conclude that PKCζ on activation changes its conformation resulting in elution from its vesicle. The downstream effect of dietary PC is to activate PKCζ, resulting in greater chylomicron output by the ER.

3.2312 Autophagic bulk sequestration of cytosolic cargo is independent of LC3, but requires GABARAPs
Szalai, P., Korseberg Hagen, L., Sætre, F., Luhr, M., Sponheim, M., Øverbye, A., Mills, I.G., Seglen, P.O. and Engedal, N.
Exp. Cell Res., ***333***, *21-38 (2015)*

LC3, a mammalian homologue of yeast Atg8, is assumed to play an important part in bulk sequestration and degradation of cytoplasm (macroautophagy), and is widely used as an indicator of this process. To critically examine its role, we followed the autophagic flux of LC3 in rat hepatocytes during conditions of maximal macroautophagic activity (amino acid depletion), combined with analyses of macroautophagic cargo sequestration, measured as transfer of the cytosolic protein lactate dehydrogenase (LDH) to sedimentable organelles. To accurately determine LC3 turnover we developed a quantitative immunoblotting procedure that corrects for differential immunoreactivity of cytosolic and membrane-associated LC3 forms, and we included cycloheximide to block influx of newly synthesized LC3. As expected, LC3 was initially degraded by the autophagic-lysosomal pathway, but, surprisingly, autophagic LC3-flux ceased after ~2 h. In contrast, macroautophagic cargo flux was well maintained, and density gradient analysis showed that sequestered LDH partly accumulated in LC3-free autophagic vacuoles. Hepatocytic macroautophagy could thus proceed independently of LC3. Silencing of either of the two mammalian Atg8 subfamilies in LNCaP prostate cancer cells exposed to macroautophagy-inducing conditions (starvation or the mTOR-inhibitor Torin1) confirmed that macroautophagic sequestration did not require the LC3 subfamily, but, intriguingly, we found the GABARAP subfamily to be essential.

3.2313 PTEN secretion in exosomes
Putz, U., Mah, S., Goh, C-P., Low, L-H., Howitt, J. and Tan, S-S.
Methods, ***77-78***, *157-163 (2015)*

PTEN was discovered as a membrane-associated tumor suppressor protein nearly two decades ago, but the concept that it can be secreted and taken up by recipient cells is revolutionary. Since then, various laboratories have reported that PTEN is indeed secreted and available for uptake by other cells in at least two different guises. First, PTEN may be packaged and exported within extracellular vesicles (EV) called exosomes. Second, PTEN may also be secreted as a naked protein in a longer isoform called PTEN-long. While the conditions favouring the secretion of PTEN-long remain unknown, PTEN secretion in exosomes is enhanced by the Ndfip1/Nedd4 ubiquitination system. In this report, we describe conditions for packaging PTEN in exosomes and their potential use for mediating non cell-autonomous functions in recipient cells. We suggest that this mode of PTEN transfer may potentially provide beneficial PTEN for tumor suppression, however it may also propagate deleterious versions of mutated PTEN causing tumorigenesis.

3.2314 Scavenger Receptor SREC-I Mediated Entry of TLR4 into Lipid Microdomains and Triggered Inflammatory Cytokine Release in RAW 264.7 Cells upon LPS Activation
Murshid, A., Gong, J., Prince, T., Borges, T.J. and Calderwood, S.K:
PloS One, ***10(4)***, *e122529 (2015)*

Scavenger receptor associated with endothelial cells I (SREC-I) was shown to be expressed in immune cells and to play a role in the endocytosis of peptides and antigen presentation. As our previous studies indicated that SREC-I required intact Toll-like receptor 4 (TLR4) expression for its functions in tumor immunity, we examined potential interactions between these two receptors. We have shown here that SREC-I became associated with TLR4 on binding bacterial lipopolysaccharides (LPS) in RAW 264.7 and

HEK 293 cells overexpressing these two receptors. The receptors then became internalized together in intracellular endosomes. SREC-I promoted TLR4-induced signal transduction through the NF-kB and MAP kinase pathways, leading to enhanced inflammatory cytokine release. Activation of inflammatory signaling through SREC-I/TLR4 complexes appeared to involve recruitment of the receptors into detergent-insoluble, cholesterol-rich lipid microdomains that contained the small GTPase Cdc42 and the non-receptor tyrosine kinase c-src. Under conditions of SREC-I activation by LPS, TLR4 activity required Cdc42 as well as cholesterol and actin polymerization for signaling through NF-kB and MAP kinase pathways in RAW 264.7 cells. SREC-I appeared to respond differently to another ligand, the molecular chaperone Hsp90 that, while triggering SREC-I-TLR4 binding caused only faint activation of the NF-kB pathway. Our experiments therefore indicated that SREC-I could bind LPS and might be involved in innate inflammatory immune responses to extracellular danger signals in RAW 264.7 cells or bone marrow-derived macrophages.

3.2315 The extracellular RNA complement of Escherichia coli

Ghosal, A., Upadhyaya, B.B., Fritz, J.V., Heintz-Buschart, A., Desai, M.S., Yusuf, D., Huang, D., Baumuratov, A., Wang, K., Galas, D. and Wilmes, P.
*MicrobiologyOpen, **4(2)**, 252-266 (2015%*

The secretion of biomolecules into the extracellular milieu is a common and well-conserved phenomenon in biology. In bacteria, secreted biomolecules are not only involved in intra-species communication but they also play roles in inter-kingdom exchanges and pathogenicity. To date, released products, such as small molecules, DNA, peptides, and proteins, have been well studied in bacteria. However, the bacterial extracellular RNA complement has so far not been comprehensively characterized. Here, we have analyzed, using a combination of physical characterization and high-throughput sequencing, the extracellular RNA complement of both outer membrane vesicle (OMV)-associated and OMV-free RNA of the enteric Gram-negative model bacterium *Escherichia coli* K-12 substrain MG1655 and have compared it to its intracellular RNA complement. Our results demonstrate that a large part of the extracellular RNA complement is in the size range between 15 and 40 nucleotides and is derived from specific intracellular RNAs. Furthermore, RNA is associated with OMVs and the relative abundances of RNA biotypes in the intracellular, OMV and OMV-free fractions are distinct. Apart from rRNA fragments, a significant portion of the extracellular RNA complement is composed of specific cleavage products of functionally important structural noncoding RNAs, including tRNAs, 4.5S RNA, 6S RNA, and tmRNA. In addition, the extracellular RNA pool includes RNA biotypes from cryptic prophages, intergenic, and coding regions, of which some are so far uncharacterised, for example, transcripts mapping to the *fimA-fimL* and *ves-spy* intergenic regions. Our study provides the first detailed characterization of the extracellular RNA complement of the enteric model bacterium *E. coli*. Analogous to findings in eukaryotes, our results suggest the selective export of specific RNA biotypes by *E. coli*, which in turn indicates a potential role for extracellular bacterial RNAs in intercellular communication.

3.2316 Subcellular localization and activation of ADAM proteases in thecontext of FasL shedding in T lymphocytes

Ebsen, H., Lettau, M., Kabelitz, D. and Janssen, O.
*Mol. Immunol., **65**, 416-428 (2015)*

The “A Disintegrin And Metalloproteinases” (ADAMs) form a subgroup of the metzincin endopeptidases. Proteolytically active members of this protein family act as sheddases and govern key processes in development and inflammation by regulating cell surface expression and release of cytokines, growth factors, adhesion molecules and their receptors. In T lymphocytes, ADAM10 sheds the death factor Fas Ligand (FasL) and thereby regulates T cell activation, death and effector function. Although FasL shedding by ADAM10 was confirmed in several studies, its regulation is still poorly defined. We recently reported that ADAM10 is highly abundant on T cells whereas its close relative ADAM17 is expressed at low levels and transiently appears at the cell surface upon stimulation. Since FasL is also stored intracellularly and brought to the plasma membrane upon stimulation, we addressed where the death factor gets exposed to ADAM proteases. We report for the first time that both ADAM10 and ADAM17 are associated with FasL-containing secretory lysosomes. Moreover, we demonstrate that TCR/CD3/CD28-stimulation induces a partial positioning of both proteases and FasL to lipid rafts and only the activation-induced raft-positioning results in FasL processing. TCR/CD3/CD28-induced FasL proteolysis is markedly affected by reducing both ADAM10 and ADAM17 protein levels, indicating that in human T cells also ADAM17 is implicated in FasL processing. Since FasL shedding is affected by cholesterol depletion and by inhibition of Src kinases or palmitoylation, we conclude that it requires mobilization and co-positioning

of ADAM proteases in lipid raft-like platforms associated with an activation of raft-associated Src-family kinases.

3.2317 Cholesterol Transport through Lysosome-Peroxisome Membrane Contacts
Chu, B-B., Liao, Y-C., Qi, W., Xie, C., Du, X., Wang, J., yang, H., Miao, H-H.., Li, B-L. and Song, B-L.
*Cell, **161**, 291-306 (2015)*

Cholesterol is dynamically transported among organelles, which is essential for multiple cellular functions. However, the mechanism underlying intracellular cholesterol transport has remained largely unknown. We established an amphotericin B-based assay enabling a genome-wide shRNA screen for delayed LDL-cholesterol transport and identified 341 hits with particular enrichment of peroxisome genes, suggesting a previously unappreciated pathway for cholesterol transport. We show dynamic membrane contacts between peroxisome and lysosome, which are mediated by lysosomal Synaptotagmin VII binding to the lipid PI(4,5)P_2 on peroxisomal membrane. LDL-cholesterol enhances such contacts, and cholesterol is transported from lysosome to peroxisome. Disruption of critical peroxisome genes leads to cholesterol accumulation in lysosome. Together, these findings reveal an unexpected role of peroxisome in intracellular cholesterol transport. We further demonstrate massive cholesterol accumulation in human patient cells and mouse model of peroxisomal disorders, suggesting a contribution of abnormal cholesterol accumulation to these diseases

3.2318 Isolation of Extracellular Vesicles for Proteomic Profiling
Choi, D-S. and Gho, Y.S:
Methods in Mol. Biol., 1295, 167-177 (2015)

Extracellular vesicles are nano-sized lipid bilayer vesicles released from most cells, including archaea, bacteria, and eukaryotic cells. These membrane vesicles play multiple roles in cell-to-cell communication, including immune modulation, angiogenesis, and transformation of cells by transferring genetic material and functional proteins. They contain specific subsets of proteins, DNA, RNA, and lipids that represent their cellular status. Furthermore, extracellular vesicles are enriched in cell type- or disease-specific vesicular proteins, especially plasma membrane proteins, which have pathophysiological functions; these vesicular proteins are considered novel diagnostic biomarkers as well as therapeutic targets. To profile the proteome, various purification methods of extracellular vesicles have been developed, but density gradient ultracentrifugation is considered the most promising. In this chapter, we describe the isolation of extracellular vesicles derived from SW480 cells and the preparation of tryptic peptides for mass-spectrometry-based proteomic analysis.

3.2319 Lateral Gene Transfer and Gene Duplication Played a Key Role in the Evolution of Mastigamoeba balamuthi Hydrogenosomes
Nyvlota, E., Stairs, C.W., Hrdy, I., Ridl, J., Mach, J., Paces, J., Roger, A.J. and tachezy, J.
*Mol. Biol. Evol., **32(4)**, 1039-1055 (2015)*

Lateral gene transfer (LGT) is an important mechanism of evolution for protists adapting to oxygen-poor environments. Specifically, modifications of energy metabolism in anaerobic forms of mitochondria (e.g., hydrogenosomes) are likely to have been associated with gene transfer from prokaryotes. An interesting question is whether the products of transferred genes were directly targeted into the ancestral organelle or initially operated in the cytosol and subsequently acquired organelle-targeting sequences. Here, we identified key enzymes of hydrogenosomal metabolism in the free-living anaerobic amoebozoan *Mastigamoeba balamuthi* and analyzed their cellular localizations, enzymatic activities, and evolutionary histories. Additionally, we characterized 1) several canonical mitochondrial components including respiratory complex II and the glycine cleavage system, 2) enzymes associated with anaerobic energy metabolism, including an unusual D-lactate dehydrogenase and acetyl CoA synthase, and 3) a sulfate activation pathway. Intriguingly, components of anaerobic energy metabolism are present in at least two gene copies. For each component, one copy possesses an mitochondrial targeting sequence (MTS), whereas the other lacks an MTS, yielding parallel cytosolic and hydrogenosomal extended glycolysis pathways. Experimentally, we confirmed that the organelle targeting of several proteins is fully dependent on the MTS. Phylogenetic analysis of all extended glycolysis components suggested that these components were acquired by LGT. We propose that the transformation from an ancestral organelle to a hydrogenosome in the *M. balamuthi* lineage involved the lateral acquisition of genes encoding extended

glycolysis enzymes that initially operated in the cytosol and that established a parallel hydrogenosomal pathway after gene duplication and MTS acquisition.

3.2320 Prometastatic NEDD9 Regulates Individual Cell Migration via Caveolin-1–Dependent Trafficking of Integrins
Kozyulina, P.Y., Loskutov, Y.V., Kozyreva, V.K: et al
Mol. Cancer Res., ***13(3)***, *423-438 (2015)*

The dissemination of tumor cells relies on efficient cell adhesion and migration, which in turn depends upon endocytic trafficking of integrins. In the current work, it was found that depletion of the prometastatic protein, NEDD9, in breast cancer cells results in a significant decrease in individual cell migration due to impaired trafficking of ligand-bound integrins. NEDD9 deficiency does not affect the expression or internalization of integrins but heightens caveolae-dependent trafficking of ligand-bound integrins to early endosomes. Increase in mobility of ligand-bound integrins is concomitant with an increase in tyrosine phosphorylation of caveolin-1 (CAV1) and volume of CAV1-vesicles. NEDD9 directly binds to CAV1 and colocalizes within CAV1 vesicles. In the absence of NEDD9, the trafficking of ligand-bound integrins from early to late endosomes is impaired, resulting in a significant decrease in degradation of ligand–integrin complexes and an increase in recycling of ligand-bound integrins from early endosomes back to the plasma membrane without ligand disengagement, thus leading to low adhesion and migration. Reexpression of NEDD9 or decrease in the amount of active, tyrosine 14 phosphorylated (Tyr14) CAV1 in NEDD9-depleted cells rescues the integrin trafficking deficiency and restores cellular adhesion and migration capacity. Collectively, these findings indicate that NEDD9 orchestrates trafficking of ligand-bound integrins through the attenuation of CAV1 activity.

3.2321 Inhibition of CD40-Induced N-Ras Activation Reduces Leishmania major Infection
Chakraborty, S., Srivastava, A., Jha, M.K., Nair, A., Pandey, S.P., Srivastava, N., Kumari, S., Singh, S., Krishnasastry, M.V. and Saha, B.
J. Immunol., ***194(8)***, *3852-3860 (2015)*

Leishmania major is a parasite that resides and replicates in macrophages. We previously showed that the parasite enhanced CD40-induced Raf-MEK-ERK signaling but inhibited PI3K-MKK-p38MAPK signaling to proleishmanial effects. As Raf and PI3K have a Ras-binding domain but exert opposite effects on *Leishmania* infection, we examined whether Ras isoforms had differential roles in *Leishmania* infection. We observed that *L. major* enhanced N-Ras and H-Ras expression but inhibited K-Ras expression in macrophages. *L. major* infection enhanced N-Ras activity but inhibited H-Ras and K-Ras activity. TLR2 short hairpin RNA or anti-TLR2 or anti-lipophosphoglycan Abs reversed the *L. major*–altered N-Ras and K-Ras expressions. Pam_3CSK_4, a TLR2 ligand, enhanced N-Ras expression but reduced K-Ras expression, indicating TLR2-regulated Ras expression in *L. major* infection. Whereas N-Ras silencing reduced *L. major* infection, K-Ras and H-Ras silencing enhanced the infection both in macrophages in vitro and in C57BL/6 mice. BALB/c-derived macrophages transduced with lentivirally expressed N-Ras short hairpin RNA and pulsed with *L. major*–expressed MAPK10 enhanced MAPK10-specific Th1-type response. CD40-deficient mice primed with these macrophages had reduced *L. major* infection, accompanied by higher IFN-γ but less IL-4 production. As N-Ras is activated by Sos, a guanine nucleotide exchange factor, we modeled the N-Ras–Sos interaction and designed two peptides from their interface. Both the cell-permeable peptides reduced *L. major* infection in BALB/c mice but not in CD40-deficient mice. These data reveal the *L. major*–enhanced CD40-induced N-Ras activation as a novel immune evasion strategy and the potential for Ras isoform–targeted antileishmanial immunotherapy and immunoprophylaxis.

3.2322 Impaired maturation of large dense-core vesicles in muted-deficient adrenal chromaffin cells
Hao, Z., Wei, L., Feng, Y., Chen, X., Du, W., Ma, J., Zhou, Z., Chen, L. and Li, W.
J. Cell Sci., ***128(7)***, *1365-1374 (2015)*

The large dense-core vesicle (LDCV), a type of lysosome-related organelle, is involved in the secretion of hormones and neuropeptides in specialized secretory cells. The granin family is a driving force in LDCV biogenesis, but the machinery for granin sorting to this biogenesis pathway is largely unknown. The *mu* mutant mouse, which carries a spontaneous null mutation on the *Muted* gene (also known as *Bloc1s5*), which encodes a subunit of the biogenesis of lysosome-related organelles complex-1 (BLOC-1), is a mouse model of Hermansky–Pudlak syndrome. Here, we found that LDCVs were enlarged in *mu* adrenal chromaffin cells. Chromogranin A (CgA, also known as CHGA) was increased in *mu* adrenals and muted-knockdown cells. The increased CgA in *mu* mice was likely due a failure to export this molecule out of

immature LDCVs, which impairs LDCV maturation and docking. In *mu* chromaffin cells, the size of readily releasable pool and the vesicle release frequency were reduced. Our studies suggest that the muted protein is involved in the selective export of CgA during the biogenesis of LDCVs.

3.2323 Outer membrane vesicles are vehicles for the delivery of Vibrio tasmaniensis virulence factors to oyster immune cells

Vanhove, A.S., Duperthuy, M., Charriere, G.M., Le Roux, F., Goudenege, D., Gourbal, B., Kieffer-Jaquinod, S., Coute, Y., Wai, S.N. and Destoumieux-garcon, D.
*Environment. Microbiol.,**17(4)**, 1152-1165 (2015)*

Vibrio tasmaniensis LGP32, a facultative intracellular pathogen of oyster haemocytes, was shown here to release outer membrane vesicles (OMVs) both in the extracellular milieu and inside haemocytes. Intracellular release of OMVs occurred inside phagosomes of intact haemocytes having phagocytosed few vibrios as well as in damaged haemocytes containing large vacuoles heavily loaded with LGP32. The OMV proteome of LGP32 was shown to be rich in hydrolases (25%) including potential virulence factors such as proteases, lipases, phospholipases, haemolysins and nucleases. One major caseinase/gelatinase named Vsp for vesicular serine protease was found to be specifically secreted through OMVs in which it is enclosed. Vsp was shown to participate in the virulence phenotype of LGP32 in oyster experimental infections. Finally, OMVs were highly protective against antimicrobial peptides, increasing the minimal inhibitory concentration of polymyxin B by 16-fold. Protection was conferred by OMV titration of polymyxin B but did not depend on the activity of Vsp or another OMV-associated protease. Altogether, our results show that OMVs contribute to the pathogenesis of LGP32, being able to deliver virulence factors to host immune cells and conferring protection against antimicrobial peptides.

3.2324 Inhibition of iron uptake by ferristatin II is exerted through internalization of DMT1 at the plasma membrane

Yanatori, I., Yasui, Y., Noguchi, Y. and Kishi, F.
*Cell Biol. Int., **39(4)**, 427-434 (2015)*

Ferristatin II, discovered as an iron transport inhibitor, promotes the internalization and degradation of transferrin receptor 1 (TfR1). DMT1, which mediates iron transport across cell membranes, is located at the plasma membrane of enterocytes and imports dietary iron into the cytosol. TfR1 is not directly engaged in the intestinal absorption of free iron, and iron uptake by DMT1 is attenuated by ferristatin II treatment. In this study, we found another function for ferristatin II in iron uptake. Ferristatin II did not cause degradation of DMT1 but did induce DMT1 internalization from the plasma membrane. Dynasore, a small molecule inhibitor of dynamin, did not inhibit this internalization by ferristatin II, which might occur via a clathrin-independent pathway.

3.2325 The mGluR5 Positive Allosteric Modulator CDPPB Inhibits SO2-Induced Protein Radical Formation and Mitochondrial Dysfunction Through Activation of Akt in Mouse Hippocampal HT22 Cells

Guan, D-F., Ren, P-Y., Hu, W. and Zhang, Y-L.
*Cell. Mol. Neurobiol., **35**, 573-583 (2015)*

Sulfur dioxide (SO_2) is a common gas pollutant that is detrimental to many organs. Previous studies have shown that SO_2 exposure is involved in neurotoxicity and increased risk of many brain disorders; however, our understanding of the mechanisms underlying SO_2-induced cytotoxicity on neuronal cells remains elusive. The group I metabotropic glutamate receptor 5 (mGluR5) can modulate addiction, pain, and neuronal cell death. In the present study, we showed that SO_2 derivatives exposure induced protein radical formation, mitochondrial dysfunction, and apoptotic cell death in neuronal HT22 cells. Pretreatment with 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl) (CDPPB), a positive allosteric modulator of mGluR5, significantly attenuated SO_2-induced neurotoxicity, which was fully prevented by the mGluR5 antagonist MPEP. CDPPB reduced the protein radical formation and inducible nitric oxide synthase (iNOS)-derived generation of nitric oxide, and inhibited mitochondrial dysfunction in both HT22 cells and isolated mitochondria after SO_2 treatment. Moreover, CDPPB increased the activation of Akt in the presence and absence of SO_2 treatment. Blocking Akt activation using the selective inhibitor LY294002 partially reversed the CDPPB-induced protection against SO_2-induced neurotoxicity. This study provides mechanistic experimental support for oxidative stress and mitochondrial dysfunction after SO_2 exposure in neuronal cells, and also introduces a novel therapeutic approach for SO_2-induced neurotoxicity.

3.2326 Lipid rafts and raft-mediated supramolecular entities in the regulation of CD95 death receptor apoptotic signaling

Gajate, C. and Mollinedo, F.

*Apoptosis, **20**, 584-606 (2015)*

Membrane lipid rafts are highly ordered membrane domains enriched in cholesterol, sphingolipids and gangliosides that have the property to segregate and concentrate proteins. Lipid and protein composition of lipid rafts differs from that of the surrounding membrane, thus providing sorting platforms and hubs for signal transduction molecules, including CD95 death receptor-mediated signaling. CD95 can be recruited to rafts in a reversible way through S-palmitoylation following activation of cells with its physiological cognate ligand as well as with a wide variety of inducers, including several antitumor drugs through ligand-independent intracellular mechanisms. CD95 translocation to rafts can be modulated pharmacologically, thus becoming a target for the treatment of apoptosis-defective diseases, such as cancer. CD95-mediated signaling largely depends on protein–protein interactions, and the recruitment and concentration of CD95 and distinct downstream apoptotic molecules in membrane raft domains, forming raft-based supramolecular entities that act as hubs for apoptotic signaling molecules, favors the generation and amplification of apoptotic signals. Efficient CD95-mediated apoptosis involves CD95 and raft internalization, as well as the involvement of different subcellular organelles. In this review, we briefly summarize and discuss the involvement of lipid rafts in the regulation of CD95-mediated apoptosis that may provide a new avenue for cancer therapy.

3.2327 Alterations in Cholesterol and Ganglioside GM1 Content of Lipid Rafts in Platelets From Patients With Alzheimer Disease

Liu, L., Zhang, K., Tan, L., Chen, Y-H. and Cao, Y-P.

*Alzheimer. Dis. Assoc. Disord., **29**, 63-69 (2015)*

The aim of this study was to investigate the changes in the protein, cholesterol, and ganglioside GM1 content of lipid rafts in platelets from patients with Alzheimer disease (AD), and identify potential blood biomarkers of the disease. A total of 31 Chinese patients with AD and 31 aged-matched control subjects were selected. Lipid rafts were isolated from platelets using Optiprep gradient centrifugation. The protein content of lipid rafts was evaluated using Micro BCA assay, the cholesterol content using molecular probes, ganglioside GM1 content using colorimetry and dot-blotting analysis. The results showed that the cholesterol and ganglioside GM1 content of lipid rafts from platelets was significantly higher in patients with AD than aged-matched control subjects, whereas the protein content of lipid rafts did not show any differences between the 2 groups. These results indicate that the increases in the cholesterol and ganglioside GM1 content of lipid rafts from the platelets of patients with AD might serve as a biochemical adjunct to the clinical diagnosis of AD.

3.2328 Delivery of liposomal contents to outer membrane vesicles from gram negative bacteria

Ficurilli, M., Liu, C., Riviello, C., Pozo, M.J. and Meers, P.R.

*Biophys. J., **108(2)**, Suppl. 1 408a (2015)*

Gram negative bacteria produce small ~50-200 nm vesicles from their outer membranes. These outer membrane vesicles (OMV) have been implicated in activities such as transmission of virulence factors, horizontal gene transfer and development of biofilms. In this investigation, we continue our studies on the association and/or fusion of various liposomes with OMV. The delivery of large encapsulated molecules into OMV from L. enzymogenes C3 was investigated using liposomes with lipid compositions previously observed to be apparently fusogenic (Bartos et al., Biophys. J. 104(2) suppl1, 90a). Liposomes (100 nm) composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(10-racglycerol) (POPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) in a 1:3 ratio or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were used to encapsulate dextran conjugates of Texas red averaging 40 kDa. They were incubated with Lysobacter OMV (30_C, 1 hr.), then sedimented through 15% iodixanol, and the fluorescence monitored as indicatiive of transfer of liposomal contents to fused products. Both liposomal compositions showed significant evidence of dextran transfer. Because biofilms also contain OMV, the interaction of these liposomes with E. coli (DH10B) biofilms was also investigated via fluorescence microscopy. Significant penetration and binding within the biofilm mass was observed, as well as possible fusion with OMV, and rarely, evidence of transfer of dextran into whole bacterial cells. Fluorescence resonance energy transfer (FRET)-based assays also demonstrated that liposomes as small as 30 nm could rapidly fuse with Lysobacter OMV, suggesting possible delivery to OMV with smaller perturbation and better biofilm penetration.

3.2329 Three-Dimensional Architecture of Murine Rod Cilium Revealed by Cryo-EM
Wensel, T. and Gilliam, J.C.
Methods in Mol. Biol., ***1271***, *267-292 (2015)*

The connecting cilium of the rod photoreceptor is a tubular structure that bridges two adjacent cellular compartments, the inner segment, the major site of biosynthesis and energy metabolism, and the outer segment, a highly specialized ciliary structure responsible for phototransduction. The connecting cilium allows for active processes of protein sorting and transport to occur between them. Mutations affecting the cargo, their transporters, and the structural components of the primary cilium and basal body lead to aberrant trafficking and photoreceptor cell death. Understanding the overall design of the cilium, its architectural organization, and the function of varied protein complexes within the structural hierarchy of the cilium requires techniques for visualizing their native three-dimensional structures at high magnification. Here we describe methods for isolating retinas from mice, purifying fragments of rod cells that include much of the inner segment and the rod photoreceptor cilia, vitrifying the cell fragments, and determining their structures by cryo-electron tomography.

3.2330 Structure-Based Mutational Analysis of Several Sites in the E Protein: Implications for Understanding the Entry Mechanism of Japanese Encephalitis Virus
Liu, H., Liu, Y., Wang, S., Zhang, Y., Zu, X., Zhou, Z., Zhang, B. and Xiao, G.-
J. Virol., ***89(10)***, *5668-5686 (2015)*

Japanese encephalitis virus (JEV), which causes viral encephalitis in humans, is a serious risk to global public health. The JEV envelope protein mediates the viral entry pathway, including receptor-binding and low-pH-triggered membrane fusion. Utilizing mutagenesis of a JEV infectious cDNA clone, mutations were introduced into the potential receptor-binding motif or into residues critical for membrane fusion in the envelope protein to systematically investigate the JEV entry mechanism. We conducted experiments evaluating infectious particle, recombinant viral particle, and virus-like particle production and found that most mutations impaired virus production. Subcellular fractionation confirmed that five mutations—in I_0, ij, BC, and FG and the R9A substitution—impaired virus assembly, and the assembled virus particles of another five mutations—in kl and the E373A, F407A, L221S, and W217A substitutions—were not released into the secretory pathway. Next, we examined the entry activity of six mutations yielding infectious virus. The results showed N154 and the DE loop are not the only or major receptor-binding motifs for JEV entry into BHK-21 cells; four residues, H144, H319, T410, and Q258, participating in the domain I (DI)-DIII interaction or zippering reaction are important to maintain the efficiency of viral membrane fusion. By continuous passaging of mutants, adaptive mutations from negatively charged amino acids to positively charged or neutral amino acids, such as E138K and D389G, were selected and could restore the viral entry activity.

3.2331 A basis for vaccine development: Comparative characterization of Haemophilus influenzae outer membrane vesicles
Roier, S., Blume, T., Klug, L., Wagner, G.E., Elhenawy, W., Zangger, K., Prassl, R., reidl, J., Daum, G., Feldman, M.F. and Schild, S.
Int. J. Med. Microbiol., ***305***, *298-309 (2015)*

Outer membrane vesicles (OMVs) are spherical and bilayered particles that are naturally released from the outer membrane (OM) of Gram-negative bacteria. They have been proposed to possess several biological roles in pathogenesis and interbacterial interactions. Additionally, OMVs have been suggested as potential vaccine candidates against infections caused by pathogenic bacteria like *Haemophilus influenzae*, a human pathogen of the respiratory tract. Unfortunately, there is still a lack of fundamental knowledge regarding OMV biogenesis, protein sorting into OMVs, OMV size and quantity, as well as OMV composition in *H. influenzae*. Thus, this study comprehensively characterized and compared OMVs and OMs derived from heterologous encapsulated as well as nonencapsulated *H. influenzae* strains. Semiquantitative immunoblot analysis revealed that certain OM proteins are enriched or excluded in OMVs suggesting the presence of regulated protein sorting mechanisms into OMVs as well as interconnected OMV biogenesis mechanisms in *H. influenzae*. Nanoparticle tracking analysis, transmission electron microscopy, as well as protein and lipooligosaccharide quantifications demonstrated that heterologous *H. influenzae* strains differ in their OMV size and quantity. Lipidomic analyses identified palmitic acid as the most abundant fatty acid, while phosphatidylethanolamine was found to be the most dominant phospholipid present in OMVs and the OM of all strains tested. Proteomic analysis confirmed that *H. influenzae* OMVs contain vaccine candidate

proteins as well as important virulence factors. These findings contribute to the understanding of OMV biogenesis as well as biological roles of OMVs and, in addition, may be important for the future development of OMV based vaccines against *H. influenzae* infections.

3.2332 Exosomes Are Unlikely Involved in Intercellular Nef Transfer
Luo, X., Fan, Y., park, I-W. and He, J.J.
*PloS One, **10(4)**, e0124436 (2015)*

HIV-1 Nef is an important pathogenic factor for HIV/AIDS pathogenesis. Several recent studies including ours have demonstrated that Nef can be transferred to neighboring cells and alters the function of these cells. However, how the intercellular Nef transfer occurs is in dispute. In the current study, we attempted to address this important issue using several complementary strategies, a panel of exosomal markers, and human CD4+ T lymphocyte cell line Jurkat and a commonly used cell line 293T. First, we showed that Nef was transferred from Nef-expressing or HIV-infected Jurkat to naïve Jurkat and other non-Jurkat cells and that the transfer required the membrane targeting function of Nef and was cell density-dependent. Then, we showed that Nef transfer was cell-cell contact-dependent, as exposure to culture supernatants or exosomes from HIV-infected Jurkat or Nef-expressing Jurkat and 293T led to little Nef detection in the target cells Jurkat. Thirdly, we demonstrated that Nef was only detected to be associated with HIV virions but not with acetylcholinesterase (AChE+) exosomes from HIV-infected Jurkat and not in the exosomes from Nef-expressing Jurkat. In comparison, when it was over-expressed in 293T, Nef was detected in detergent-insoluble AChE+/CD81^{low}/TSG101^{low} exosomes, but not in detergent-soluble AChE-/CD81^{high}/TSG101^{high} exosomes. Lastly, microscopic imaging showed no significant Nef detection in exosomal vesicle-like structures in and out 293T. Taken together, these results show that exosomes are unlikely involved in intercellular Nef transfer. In addition, this study reveals existence of two types of exosomes: AChE+/CD81^{low}/TSG101^{low} exosomes and AChE-/CD81^{high}/TSG101^{high} exosomes.

3.2333 Endomembrane proteomics reveals putative enzymes involved in cell wall metabolism in wheat grain outer layers
Chateigner-Boutin, A-L., Suliman, M., Bouchet, B., Alvarado, C., Lollier, V., Rogniaux, H., Guillon, F. and Larre, C.
*J. Exp. Botany, **66(9)**, 2649-2658 (2015)*

Cereal grain outer layers fulfil essential functions for the developing seed such as supplying energy and providing protection. In the food industry, the grain outer layers called 'the bran' is valuable since it is rich in dietary fibre and other beneficial nutriments. The outer layers comprise several tissues with a high content in cell wall material. The cell wall composition of the grain peripheral tissues was investigated with specific probes at a stage of active cell wall synthesis. Considerable wall diversity between cell types was revealed. To identify the cellular machinery involved in cell wall synthesis, a subcellular proteomic approach was used targeting the Golgi apparatus where most cell wall polysaccharides are synthesized. The tissues were dissected into outer pericarp and intermediate layers where 822 and 1304 proteins were identified respectively. Many carbohydrate-active enzymes were revealed: some in the two peripheral grain fractions, others only in one tissue. Several protein families specific to one fraction and with characterized homologs in other species might be related to the specific detection of a polysaccharide in a particular cell layer. This report provides new information on grain cell walls and its biosynthesis in the valuable outer tissues, which are poorly studied so far. A better understanding of the mechanisms controlling cell wall composition could help to improve several quality traits of cereal products (e.g. dietary fibre content, biomass conversion to biofuel).

3.2334 The pathogenic human Torsin A in Drosophila activates the unfolded protein response and increases susceptibility to oxidative stress
Kim, A-Y., Seo, J-B., Kim, W-t., Choi, H.J., Kim, S-Y., Morrow, G., Tanguya, R.M., Steller, H. and Koh, Y.H.
*BMC Genomics, **16**:338 (2015)*

Background
Dystonia1 (DYT1) dystonia is caused by a glutamic acid deletion (ΔE) mutation in the gene encoding Torsin A in humans (HTorA). To investigate the unknown molecular and cellular mechanisms underlying DYT1 dystonia, we performed an unbiased proteomic analysis.
Results

We found that the amount of proteins and transcripts of an Endoplasmic reticulum (ER) resident chaperone Heat shock protein cognate 3 (HSC3) and a mitochondria chaperone Heat Shock Protein 22 (HSP22) were significantly increased in the HTorA$^{\Delta E}$– expressing brains compared to the normal HTorA (HTorA^{WT}) expressing brains. The physiological consequences included an increased susceptibility to oxidative and ER stress compared to normal HTorA^{WT} flies. The alteration of transcripts of Inositol-requiring enzyme-1 (IRE1)-dependent spliced X box binding protein 1(Xbp1), several ER chaperones, a nucleotide exchange factor, Autophagy related protein 8b (ATG8b) and components of the ER associated degradation (ERAD) pathway and increased expression of the Xbp1-enhanced Green Fluorescence Protein (eGFP) in HTorA$^{\Delta E}$ brains strongly indicated the activation of the unfolded protein response (UPR). In addition, perturbed expression of the UPR sensors and inducers in the HTorA$^{\Delta E}$ *Drosophila* brains resulted in a significantly reduced life span of the flies. Furthermore, the types and quantities of proteins present in the anti-HSC3 positive microsomes in the HTorA$^{\Delta E}$ brains were different from those of the HTorA^{WT} brains.

Conclusion

Taken together, these data show that HTorA$^{\Delta E}$ in *Drosophila* brains may activate the UPR and increase the expression of HSP22 to compensate for the toxic effects caused by HTorA$^{\Delta E}$ in the brains.

3.2335 A Voltage-Gated Calcium Channel Regulates Lysosomal Fusion with Endosomes and Autophagosomes and Is Required for Neuronal Homeostasis

Tian, X., Gala, U., Zhang, Y., Shang, W., jaiswal, SD.N., di Ronza, A., Jaiswal, M., Yamamoto, S., Sandoval, H., Duraine, L., Sardiello, M., Sillitoe, R.V., Ventatachalam, K., Fan, H., Bellen, H.J: and Tong, C.

*PloS Biology, **13(3)**, e1002103 (2015)*

Autophagy helps deliver sequestered intracellular cargo to lysosomes for proteolytic degradation and thereby maintains cellular homeostasis by preventing accumulation of toxic substances in cells. In a forward mosaic screen in *Drosophila* designed to identify genes required for neuronal function and maintenance, we identified multiple *cacophony (cac)* mutant alleles. They exhibit an age-dependent accumulation of autophagic vacuoles (AVs) in photoreceptor terminals and eventually a degeneration of the terminals and surrounding glia. *cac* encodes an α1 subunit of a *Drosophila* voltage-gated calcium channel (VGCC) that is required for synaptic vesicle fusion with the plasma membrane and neurotransmitter release. Here, we show that *cac* mutant photoreceptor terminals accumulate AV-lysosomal fusion intermediates, suggesting that Cac is necessary for the fusion of AVs with lysosomes, a poorly defined process. Loss of another subunit of the VGCC, α2δ or *straightjacket (stj)*, causes phenotypes very similar to those caused by the loss of *cac*, indicating that the VGCC is required for AV-lysosomal fusion. The role of VGCC in AV-lysosomal fusion is evolutionarily conserved, as the loss of the mouse homologues, *Cacna1a* and *Cacna2d2*, also leads to autophagic defects in mice. Moreover, we find that CACNA1A is localized to the lysosomes and that loss of lysosomal *Cacna1a* in cerebellar cultured neurons leads to a failure of lysosomes to fuse with endosomes and autophagosomes. Finally, we show that the lysosomal CACNA1A but not the plasma-membrane resident CACNA1A is required for lysosomal fusion. In summary, we present a model in which the VGCC plays a role in autophagy by regulating the fusion of AVs with lysosomes through its calcium channel activity and hence functions in maintaining neuronal homeostasis.

3.2336 MPP1 as a Factor Regulating Phase Separation in Giant Plasma Membrane-Derived Vesicles

Podkalicka, J., Biernatowska, A., Majkowski, M., Grzybek, M. and Sikorski, A.F.

*Biophys. J., **108**, 2201-2211 (2015)*

The existence of membrane-rafts helps to conceptually understand the spatiotemporal organization of membrane-associated events (signaling, fusion, fission, etc.). However, as rafts themselves are nanoscopic, dynamic, and transient assemblies, they cannot be directly observed in a metabolizing cell by traditional microscopy. The observation of phase separation in giant plasma membrane-derived vesicles from live cells is a powerful tool for studying lateral heterogeneity in eukaryotic cell membranes, specifically in the context of membrane rafts. Microscopic phase separation is detectable by fluorescent labeling, followed by cooling of the membranes below their miscibility phase transition temperature. It remains unclear, however, if this lipid-driven process is tuneable in any way by interactions with proteins. Here, we demonstrate that MPP1, a member of the MAGUK family, can modulate membrane properties such as the fluidity and phase separation capability of giant plasma membrane-derived vesicles. Our data suggest that physicochemical domain properties of the membrane can be modulated, without major changes in lipid composition, through proteins such as MPP1.

3.2337 Prostaglandins regulate nuclear localization of Fascin and its function in nucleolar architecture
Groen, C.M., Jayo, A., parsons, M. and Tottle, T.L.
*Mol. Biol. Cell, **26**, 1901-1917 (2015)*

Fascin, a highly conserved actin-bundling protein, localizes and functions at new cellular sites in both *Drosophila* and multiple mammalian cell types. During *Drosophila* follicle development, in addition to being cytoplasmic, Fascin is in the nuclei of the germline-derived nurse cells during stages 10B–12 (S10B–12) and at the nuclear periphery during stage 13 (S13). This localization is specific to Fascin, as other actin-binding proteins, Villin and Profilin, do not exhibit the same subcellular distribution. In addition, localization of fascin1 to the nucleus and nuclear periphery is observed in multiple mammalian cell types. Thus the regulation and function of Fascin at these new cellular locations is likely to be highly conserved. In *Drosophila*, loss of prostaglandin signaling causes a global reduction in nuclear Fascin and a failure to relocalize to the nuclear periphery. Alterations in nuclear Fascin levels result in defects in nucleolar morphology in both *Drosophila* follicles and cultured mammalian cells, suggesting that nuclear Fascin plays an important role in nucleolar architecture. Given the numerous roles of Fascin in development and disease, including cancer, our novel finding that Fascin has functions within the nucleus sheds new light on the potential roles of Fascin in these contexts.

3.2338 Muscle Releases Alpha-Sarcoglycan Positive Extracellular Vesicles Carrying miRNAs in the Bloodstream
Guescini, M., Canonico, B., Lucertini, F., Maggio, S., Annibalini, G., Barbieri, E., Luchetti, F., Papa, S. and Stocchi, V.
*PloS One, **10(5)**, e0125094 (2015)*

In the past few years, skeletal muscle has emerged as an important secretory organ producing soluble factors, called myokines, that exert either autocrine, paracrine or endocrine effects. Moreover, recent studies have shown that muscle releases microRNAs into the bloodstream in response to physical exercise. These microRNAs affect target cells, such as hormones and cytokines. The mechanisms underlying microRNA secretion are poorly characterized at present. Here, we investigated whether muscle tissue releases extracellular vesicles (EVs), which carry microRNAs in the bloodstream under physiological conditions such as physical exercise. Using density gradient separation of plasma from sedentary and physically fit young men we found EVs positive for TSG101 and alpha-sarcoglycan (SGCA), and enriched for miR-206. Cytometric analysis showed that the SGCA+ EVs account for 1–5% of the total and that 60–65% of these EVs were also positive for the exosomal marker CD81. Furthermore, the SGCA-immuno captured sub-population of EVs exhibited higher levels of the miR-206/miR16 ratio compared to total plasma EVs. Finally, a significant positive correlation was found between the aerobic fitness and muscle-specific miRNAs and EV miR-133b and -181a-5p were significantly up-regulated after acute exercise. Thus, our study proposes EVs as a novel means of muscle communication potentially involved in muscle remodeling and homeostasis.

3.2339 Generation of nanovesicles with sliced cellular membrane fragments for exogenous material delivery
Yoon, J., Jo, W., Jeong, D., Kim, J., jeong, H. and Park, J.
*Biomaterials, **59**, 12-20 (2015)*

We propose a microfluidic system that generates nanovesicles (NVs) by slicing living cell membrane with microfabricated 500 nm-thick silicon nitride (Si_xN_y) blades. Living cells were sliced by the blades while flowing through microchannels lined with the blades. Plasma membrane fragments sliced from the cells self-assembled into spherical NVs of ~100–300 nm in diameter. During self-assembly, the plasma membrane fragments enveloped exogenous materials (here, polystyrene latex beads) from the buffer solution. About 30% of beads were encapsulated in NVs, and the generated NVs delivered the encapsulated beads across the plasma membrane of recipient cells, but bare beads could not penetrate the plasma membrane of recipient cells. This result implicates that the NVs generated using the method in this study can encapsulate and deliver exogenous materials to recipient cells, whereas exosomes secreted by cells can deliver only endogenous cellular materials.

3.2340 Yeast Coq9 controls deamination of coenzyme Q intermediates that derive from para-aminobenzoic acid
He, C.H., Black, D.S., Nguyen, T.P.T., Wang, C., Srinivasan, C. and Clarke, C.F.
*Biochim. Biophys. Acta, **1851**, 1227-1239 (2015)*

Coq9 is a polypeptide subunit in a mitochondrial multi-subunit complex, termed the CoQ-synthome, required for biosynthesis of coenzyme Q (ubiquinone or Q). Deletion of *COQ9* results in dissociation of the CoQ-synthome, but over-expression of Coq8 putative kinase stabilizes the CoQ-synthome in the *coq9* null mutant and leads to the accumulation of two nitrogen-containing Q intermediates, imino-demethoxy-Q_6($IDMQ_6$) and 3-hexaprenyl-4-aminophenol (4-AP) when *para*-aminobenzoic acid (pABA) is provided as a ring precursor. To investigate whether Coq9 is responsible for deamination steps in Q biosynthesis, we utilized the yeast *coq5-5* point mutant. The yeast *coq5-5* point mutant is defective in the *C*-methyltransferase step of Q biosynthesis but retains normal steady-state levels of the Coq5 polypeptide. Here, we show that when high amounts of $^{13}C_6$-pABA are provided, the *coq5-5* mutant accumulates both$^{13}C_6$-imino-demethyl-demethoxy-Q_6 ($^{13}C_6$-$IDDMQ_6$) and $^{13}C_6$-demethyl-demethoxy-Q_6 ($^{13}C_6$-$DDMQ_6$). Deletion of *COQ9* in the yeast *coq5-5* mutant along with Coq8 over-expression and $^{13}C_6$- pABA labeling leads to the absence of $^{13}C_6$-$DDMQ_6$, and the nitrogen-containing intermediates $^{13}C_6$-4-AP and $^{13}C_6$-$IDDMQ_6$ persist. We describe a *coq9* temperature-sensitive mutant and show that at the non-permissive temperature, steady-state polypeptide levels of Coq9-ts19 increased, while Coq4, Coq5, Coq6, and Coq7 decreased. The *coq9-ts19* mutant had decreased Q_6 content and increased levels of nitrogen-containing intermediates. These findings identify Coq9 as a multi-functional protein that is required for the function of Coq6 and Coq7 hydroxylases, for removal of the nitrogen substituent from pABA-derived Q intermediates, and is an essential component of the CoQ synthome.

3.2341 Characterisation of detergent-insoluble membranes in pollen tubes of Nicotiana tabacum (L.)
Moscatelli, A., Gagliardi, A., Maneta-Peyret, L., Bini, L., Stroppa, N., Onelli, E., Landi, C., Scali, M., Idilli, A.I. and Moreau, P.
Biology Open, 4, 378-399 (2015)

Pollen tubes are the vehicle for sperm cell delivery to the embryo sac during fertilisation of Angiosperms. They provide an intriguing model for unravelling mechanisms of growing to extremes. The asymmetric distribution of lipids and proteins in the pollen tube plasma membrane modulates ion fluxes and actin dynamics and is maintained by a delicate equilibrium between exocytosis and endocytosis. The structural constraints regulating polarised secretion and asymmetric protein distribution on the plasma membrane are mostly unknown. To address this problem, we investigated whether ordered membrane microdomains, namely membrane rafts, might contribute to sperm cell delivery. Detergent insoluble membranes, rich in sterols and sphingolipids, were isolated from tobacco pollen tubes. MALDI TOF/MS analysis revealed that actin, prohibitins and proteins involved in methylation reactions and in phosphoinositide pattern regulation are specifically present in pollen tube detergent insoluble membranes. Tubulins, voltage-dependent anion channels and proteins involved in membrane trafficking and signalling were also present. This paper reports the first evidence of membrane rafts in Angiosperm pollen tubes, opening new perspectives on the coordination of signal transduction, cytoskeleton dynamics and polarised secretion.

3.2342 Mitochondria of a human multidrug-resistant hepatocellular carcinoma cell line constitutively express inducible nitric oxide synthase in the inner membrane
Fantappie, O., Sassoli, C., Tani, A., Nosi, D., Marchatti, S., Formigli, L. and Mazzanti, R.
*J. Cell. Mol. Med., **19(6)**, 1410-1417 (2015)*

Mitochondria play a crucial role in pathways of stress conditions. They can be transported from one cell to another, bringing their features to the cell where they are transported. It has been shown in cancer cells overexpressing multidrug resistance (MDR) that mitochondria express proteins involved in drug resistance such as P-glycoprotein (P-gp), breast cancer resistant protein and multiple resistance protein-1. The MDR phenotype is associated with the constitutive expression of COX-2 and iNOS, whereas celecoxib, a specific inhibitor of COX-2 activity, reverses drug resistance of MDR cells by releasing cytochrome c from mitochondria. It is possible that COX-2 and iNOS are also expressed in mitochondria of cancer cells overexpressing the MDR phenotype. This study involved experiments using the human HCC PLC/PRF/5 cell line with and without MDR phenotype and melanoma A375 cells that do not express the MDR1 phenotype but they do iNOS. Western blot analysis, confocal immunofluorescence and immune electron microscopy showed that iNOS is localized in mitochondria of MDR1-positive cells, whereas COX-2 is not. Low and moderate concentrations of celecoxib modulate the expression of iNOS and P-gp in mitochondria of MDR cancer cells independently from inhibition of COX-2 activity. However, A375 cells that express iNOS also in mitochondria, were not MDR1 positive. In conclusion, iNOS can be localized in mitochondria of HCC cells overexpressing MDR1 phenotype, however this phenomenon appears

independent from the MDR1 phenotype occurrence. The presence of iNOS in mitochondria of human HCC cells phenotype probably concurs to a more aggressive behaviour of cancer cells.

3.2343 Proteomics of Aggregatibacter actinomycetemcomitans Outer Membrane Vesicles
Kieselbach, T., Zijnge, V., Granström, E. and Oscarsson, J.
*PloS One, **10(9)**, e0138591 (2015)*

Aggregatibacter actinomycetemcomitans is an oral and systemic pathogen associated with aggressive forms of periodontitis and with endocarditis. Outer membrane vesicles (OMVs) released by this species have been demonstrated to deliver effector proteins such as cytolethal distending toxin (CDT) and leukotoxin (LtxA) into human host cells and to act as triggers of innate immunity upon carriage of NOD1- and NOD2-active pathogen-associated molecular patterns (PAMPs). To improve our understanding of the pathogenicity-associated functions that *A. actinomycetemcomitans* exports via OMVs, we studied the proteome of density gradient-purified OMVs from a rough-colony type clinical isolate, strain 173 (serotype e) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This analysis yielded the identification of 151 proteins, which were found in at least three out of four independent experiments. Data are available via ProteomeXchange with identifier PXD002509. Through this study, we not only confirmed the vesicle-associated release of LtxA, and the presence of proteins, which are known to act as immunoreactive antigens in the human host, but we also identified numerous additional putative virulence-related proteins in the *A. actinomycetemcomitans* OMV proteome. The known and putative functions of these proteins include immune evasion, drug targeting, and iron/nutrient acquisition. In summary, our findings are consistent with an OMV-associated proteome that exhibits several offensive and defensive functions, and they provide a comprehensive basis to further disclose roles of *A. actinomycetemcomitans* OMVs in periodontal and systemic disease.

3.2344 K63 linked ubiquitin chain formation is a signal for HIF1A degradation by Chaperone-Mediated Autophagy
Ferreira, J.V., Soares, A.R., Ramalho, J.S., Pereira, P. and Girao, H.
*Scientific Reports, **5**:10210 (2015)*

Chaperone-Mediated Autophagy is a selective form of autophagy. Recently, the degradation of a newly identified CMA substrate, the HIF1A transcription factor, was found to be regulated by the ubiquitin ligase STUB1. In this study we show, for the first time, that K63 ubiquitination is necessary for CMA degradation of HIF1A *in vitro* and *in vivo*. Additionally, STUB1 mediates K63 linked ubiquitination of HIF1A. Our findings add a new regulatory step and increase the specificity of the molecular mechanism involved in CMA degradation of HIF1A, expanding the role of ubiquitination to yet another biological process, since the same mechanism might be applicable to other CMA substrates.

3.2345 Directional cell movement through tissues is controlled by exosome secretion
Sung, B.H., Ketova, T., Hoshino, D., Zijlstra, A. and Weaver, A.M.
*Nature Communications, **6**:7164 (2015)*

Directional cell movement through tissues is critical for multiple biological processes and requires maintenance of polarity in the face of complex environmental cues. Here we use intravital imaging to demonstrate that secretion of exosomes from late endosomes is required for directionally persistent and efficient *in vivo* movement of cancer cells. Inhibiting exosome secretion or biogenesis leads to defective tumour cell migration associated with increased formation of unstable protrusions and excessive directional switching. *In vitro r*escue experiments with purified exosomes and matrix coating identify adhesion assembly as a critical exosome function that promotes efficient cell motility. Live-cell imaging reveals that exosome secretion directly precedes and promotes adhesion assembly. Fibronectin is found to be a critical motility-promoting cargo whose sorting into exosomes depends on binding to integrins. We propose that autocrine secretion of exosomes powerfully promotes directionally persistent and effective cell motility by reinforcing otherwise transient polarization states and promoting adhesion assembly.

3.2346 ELMOD2 is anchored to lipid droplets by palmitoylation and regulates adipocyte triglyceride lipase recruitment
Suzuki, M., Murakami, T., Cheng, J., Kano, H., Fukata, M. and Fujimoto, T.
*Mol. Biol. Cell, **26**, 2333-2342 (2015)*

Adipocyte triglyceride lipase (ATGL) is the major enzyme involved in the hydrolysis of triglycerides. The Arf1–coat protein complex I (COPI) machinery is known to be engaged in the recruitment of ATGL to lipid droplets (LDs), but the regulatory mechanism has not been clarified. In the present study, we found that ELMOD2, a putative noncanonical Arf–GTPase activating protein (GAP) localizing in LDs, plays an important role in controlling ATGL transport to LDs. We showed that knockdown of ELMOD2 by RNA interference induced an increase in the amount of ATGL existing in LDs and decreased the total cellular triglycerides. These effects of ELMOD2 knockdown were canceled by transfection of small interfering RNA-resistant cDNA of wild-type ELMOD2 but not by that of mutated ELMOD2 lacking the Arf-GAP activity. ELMOD2 was distributed in the endoplasmic reticulum and mitochondria as well as in LDs, but palmitoylation was required only for distribution to LDs. An ELMOD2 mutant deficient in palmitoylation failed to reconstitute the ATGL transport after the ELMOD2 knockdown, indicating that distribution in LDs is indispensable to the functionality of ELMOD2. These results indicate that ELMOD2 regulates ATGL transport and cellular lipid metabolism by modulating the Arf1-COPI activity in LDs.

3.2347 **Association of Cytokines With Exosomes in the Plasma of HIV-1–Seropositive Individuals**
Konadu, K.A., Chu, J., Huang, M.B., Amancha, P.K., Armstrong, W., Powell, M.D., Villinger, F. and Bond, V.C.
*Journal of Infectious Disease, **211(11)**, 1712-1716 (2015)*

Human immunodeficiency virus (HIV)-infected and viremic individuals exhibit elevated levels of plasma cytokines. Here we show that most cytokines are not in free form but appear associated with exosomes that are distinct from virions. Purified exosomes were analyzed to determine the levels of 21 cytokines and chemokines and compared with exosome-depleted plasma. Most cytokines were markedly enriched in exosomes from HIV-positive individuals relative to negative controls and to plasma. Moreover, exposure of naive peripheral blood mononuclear cells to exosomes purified from HIV-positive patients induced CD38 expression on naive and central memory $CD4^+$ and $CD8^+$ T cells, probably contributing to inflammation and viral propagation via bystander cell activation.

3.2348 **Novel steps in the autophagic-lysosomal pathway**
Sætre, F., Korseberg hagen, L., Engedal, N. and Seglen, P.O.
*FEBS J., **282(11)**, 2202-2214 (2015)*

Autophagy is the process by which portions of cytoplasm are enclosed by membranous organelles, phagophores, which deliver the sequestered cytoplasm to degradative autophagic vacuoles. Genes and proteins involved in phagophore manufacture have been extensively studied, but little is known about how mature phagophores proceed through the subsequent steps of expansion, closure and fusion. Here we have addressed these issues by combining our unique autophagic cargo sequestration assay (using the cytosolic enzyme lactate dehydrogenase as a cargo marker) with quantitative measurements of the lipidation-dependent anchorage and turnover of the phagophore-associated protein LC3. In isolated rat hepatocytes, amino acid starved to induce maximal autophagic activity, the two unrelated reversible autophagy inhibitors 3-methyladenine (3MA) and thapsigargin (TG) both blocked cargo sequestration completely. However, whereas 3MA inhibited LC3 lipidation, TG did not, thus apparently acting at a post-lipidation step to prevent phagophore closure. Intriguingly, the resumption of cargo sequestration seen upon release from a reversible TG block was completely suppressed by 3MA, revealing that 3MA not only inhibits LC3 lipidation but also (like TG) blocks phagophore closure at a post-lipidation step. 3MA did not, however, prevent the resumption of lysosomal LC3 degradation, indicating that phagophores could fuse directly with degradative autophagic vacuoles without carrying cytosolic cargo. This fusion step was clearly blocked by TG. Furthermore, density gradient centrifugation revealed that a fraction of the LC3-marked phagophores retained by TG could be density-shifted by the acidotropic drug propylamine along with the lysosomal marker cathepsin B, suggesting physical association of some phagophores with lysosomes prior to cargo sequestration.

3.2349 **Tu1405 Apolipoprotein-Av Resists Proteolysis in the Intestinal Tract and Is Absorbed Intact, Controlled by Dietary Phosphatidylcholine**
Siddiqi, S., Polly, S., Siddiqi, T. and Mansbach, C.M.
*Gastroenterology, **148(4)**, S880-S881 (2015)*

Purpose of Study: Apolipoprotein-AV (ApoAV) correlates with serum triacylglycerol (TAG) levels and mutations of the apoAV gene cause otherwise unexplained elevated TAG levels in humans. ApoAV is

present in enterocytes but is not synthesized by the intestinal cells suggesting that the apoAV is absorbed intact even though it is a 39 kDa protein. Here wetest the hypothesis that apoAV is endocytosed via caveolin-1 containing endocytic vesicles (CEV). We have proposed that CEV are the major mechanism for dietary fatty acid absorption (BBA 183; 1311, 2013). **Methods Used:** Brush borders (BB) were prepared by the Mg2+ precipitation method from rat intestine. The purity of the preparation was judged by alkaline phosphatase activity which was 17 fold that of the intestinal homogenate. Cytosol was prepared by standard methods. Caveolae were isolated from Triton X-100 treated BB and CEV from cytosol using an OptiPrep gradient. The CEV appeared in the detergent resistant fraction. Immuno-precipitation was used to identify BB and cytosolic proteins associated with apoAV *in vivo* using Triton X-100 solubilized BB and cytosol proteins. Proteins were identified by immunoblot. **Results:** In both BB and cytosol, apoAV strongly bound to CD36 and caveolin-1 in the detergent resistant fraction and weakly to clathrin and intestinal alkaline phosphatase (IAP). These results are consistent with the apoAV being on CEV. ApoAV migrated at 39 kDa on SDS-PAGE in BB and cytosol as expected for apoAV. By contrast, pancreatic cholesterol esterase (CEL), which is known to be absorbed by clathrin coated endocytic vesicles, strongly bound to clathrin and IAP and weakly to caveolin-1 and CD36 in the BB and cytosol. The cytosol from caveolin-1 knock out mice did not contain apoAV but did contain CEL. Rat intestinal fluid harvested post corn oil plus albumin gavage contained intact apoAV but greatly degraded albumin. We progressively increased the amount of phosphatidylcholine (PC) delivered to rat intestine using 5 different models. The amount of apoAV in intestinal cytosol was inversely related to the amount of PC delivered. Incubation of apoAV containing CEV with ER showed a 4 fold increase in ER-apoAV suggesting delivery of apoAV from CEV to the ER. **Conclusions:** We conclude that apoAV, unlike albumin, resists proteolysis in the intestinal tract. ApoAV is bound to caveolin-1 and CD36 on intestinal
BB and is endocytosed via CEV. The CEV transport apoAV to the ER. ApoAV's binding to BB is controlled by PC concentrations in the intestinal lumen. CEL binds to clathrin and IAP and is endocytosed via clathrin coated vesicles, separate from the CEV pathway. Cartoon of absorption of apoAV showing normal uptake via CEV, blockage of uptake by lyso-PC, and delivery of apoAV to the ER on CEV.

3.2350 Exosome release following activation of the dendritic cell immunoreceptor: A potential role in HIV-1 pathogenesis

Mfunyi, C.M., Vaillancourt, M., Vitry, J., Batomene, T-R. N., Posvandzic, A., lambert, A.A. and Gilbert, C.

*Virology, **484**, 103-112 (2015)*

Exosomes are extracellular vesicles (EVs) that play a role in intercellular communication. Stimulation of dendritic cells by the HIV-1 virus triggers their release. HIV-1 binds to dendritic cells via dendritic cell immunoreceptor (DCIR). This study shows that inhibiting the binding to DCIR significantly decreases exosome release by HIV-1-pulsed dendritic cells. In addition, exosome release from Raji-CD4 expressing DCIR cells stimulated by anti-DCIR or HIV-1 is decreased when the immunoreceptor tyrosine-based inhibition motif (ITIM) signaling motif of DCIR is mutated. Unlike the EVs released from Raji-CD4-DCIR cells after antibody stimulation, those released from HIV-1-infected cells contain the pro-apoptotic protein DAP-3. Furthermore, EVs from HIV-1 pulsed dendritic cells increase spontaneous apoptosis in uninfected CD4 T lymphocytes while they decrease it in neutrophils. This study describes for the first time that DCIR plays a role in the release of exosomes strengthening the importance of this receptor and EVs/exosomes in HIV-1 pathogenesis.

3.2351 Vitamin E: Curse or benefit in Alzheimer's disease? A systematic investigation of the impact of α-, γ- and δ-tocopherol on Aβ generation and degradation in neuroblastoma cells

Grimm, M.O.W., Stahlmann, C.P., Mett, J., Haupenthal, V.J., Zimmer, V.C., Lehmann, J., Hundsdörfer, B., Endres, K., Grimm, H.S. and Hartmann, T.

*J. Nutr. Health Aging, **19(6)**, 646-654 (2015)*

Objectives

The E vitamins are a class of lipophilic compounds including tocopherols, which have high antioxidative properties. Because of the elevated lipid peroxidation and increased reactive oxidative species in Alzheimer's disease (AD) many attempts have been made to slow down the progression of AD by utilizing the antioxidative action of vitamin E. Beside the mixed results of these studies nothing is known about the impact of vitamin E on the mechanisms leading to amyloid-β production and degradation being responsible for the plaque formation, one of the characteristic pathological hallmarks in AD. Here we systematically investigate the influence of different tocopherols on Aβ production and degradation in neuronal cell lines.

Measurements
Beside amyloid-β level the mechanisms leading to Aβ production and degradation are examined.
Results
Surprisingly, all tocopherols have shown to increase Aβ level by enhancing the Aβ production and decreasing the Aβ degradation. Aβ production is enhanced by an elevated activity of the involved enzymes, the β- and γ-secretase. These secretases are not directly affected, but tocopherols increase their protein level and expression. We could identify significant differences between the single tocopherols; whereas α-tocopherol had only minor effects on Aβ production, δ-tocopherol showed the highest potency to increase Aβ generation. Beside Aβ production, Aβ clearance was decreased by affecting IDE, one of the major Aβ degrading enzymes.
Conclusions
Our results suggest that beside the beneficial antioxidative effects of vitamin E, tocopherol has in respect to AD also a potency to increase the amyloid-β level, which differ for the analysed tocopherols. We therefore recommend that further studies are needed to clarify the potential role of these various vitamin E species in respect to AD and to identify the form which comprises an antioxidative property without having an amyloidogenic potential.

3.2352 Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles
Minciacchi, V.R. et al
*Oncotarget, **6(13)**, 11327-11341 (2015)*

Large oncosomes (LO) are atypically large (1-10μm diameter) cancer-derived extracellular vesicles (EVs), originating from the shedding of membrane blebs and associated with advanced disease. We report that 25% of the proteins, identified by a quantitative proteomics analysis, are differentially represented in large and nano-sized EVs from prostate cancer cells. Proteins enriched in large EVs included enzymes involved in glucose, glutamine and amino acid metabolism, all metabolic processes relevant to cancer. Glutamine metabolism was altered in cancer cells exposed to large EVs, an effect that was not observed upon treatment with exosomes. Large EVs exhibited discrete buoyant densities in iodixanol (OptiPrep^{TM}) gradients. Fluorescent microscopy of large EVs revealed an appearance consistent with LO morphology, indicating that these structures can be categorized as LO. Among the proteins enriched in LO, cytokeratin 18 (CK18) was one of the most abundant (within the top 5th percentile) and was used to develop an assay to detect LO in the circulation and tissues of mice and patients with prostate cancer. These observations indicate that LO represent a discrete EV type that may play a distinct role in tumor progression and that may be a source of cancer-specific markers.

3.2353 Characterization of extracellular vesicles (exosomes) from HIV-1 infected macrophages treated with HIV-1 protease inhibitor, Ritonavir.
Deshmane, S., Sheffield, J., Khalili, K. and Datta, P.
*J. Neurovirol., **21**, Suppl. 1, Abstract P37, S1-S87 82015)*

Antiretroviral therapy (ART) prevents HIV-associated neurocognitive disorders (HAND). However, milder forms of HAND are still prevalent despite widespread use of ART. Ritonavir-boosted protease inhibitor monotherapy is a maintenance strategy that prevents nucleoside reverse transcriptase inhibitor toxicity and has been effective in maintaining longterm viral suppression in the majority of patients. In this study, we assessed whether extracellular vesicles (EVs) released from HIV-1 infected monocyte/macrophages cultured in the presence of HIV-1 protease inhibitor, Ritonavir, play a role in neurodegeneration. EVs were derived from conditioned media of U937 cells and U1 cells that are latently infected with HIV-1 and cultured in the presence and absence of HIV-1 protease inhibitor, Ritonavir (2.5 and 5 micro molar) by ultracentrifugation and Iodixanol (optiprep) gradient centrifugation. The EVs were characterized for markers of exosomes such as Tsg101 and Alix, and Acetylcholinesterase enzyme (AchE) activity, viral proteins Nef and Gag, and scanning electron microscopy. We have observed significant increase in Nef and Gag proteins in exosomes derived from ritonavir treated cells. Neuronal cultures treated with U1 and U1-ritonavir exosomes were found to be severely compromised in their ability to maintain existing neuronal network as well as their ability to form neurites in a scratch-wound assay. We observed a significant down regulation of cAMP-responseelement- binding protein (CREB) phosphorylation in neurons treated with U1 exosomes and U1 exosomes derived from Ritonavir treated cells in comparison to exosomes derived from U937 cells. Furthermore, we observed significant down

regulation of CREB regulated gene expression in these neurons. Collectively, these observations demonstrate that exosomes derived from HIV-1 infected cell and cells treated with Ritonavir can cause neuronal dysfunction and degeneration by targeting CREB signaling pathway. The studies were funded by a pilot grant to PD (CNAC NIMH Grant Number P30MH092177) and ultilized services offered by core facilities of CNAC.

3.2354 Overexpression of the Insulin-Like Growth Factor II Receptor Increases β-Amyloid Production and Affects Cell Viability

Wang, Y., Buggia-Prevot, V., Zavorka, M.E., Bleackley, R.C., MacDonald, R.G., Thinakaran, G. and kar, S.

Mol. Cell. Biol., ***35(14)***, *2368-2384 (2015)*

Amyloid β (Aβ) peptides originating from amyloid precursor protein (APP) in the endosomal-lysosomal compartments play a critical role in the development of Alzheimer's disease (AD), the most common type of senile dementia affecting the elderly. Since insulin-like growth factor II (IGF-II) receptors facilitate the delivery of nascent lysosomal enzymes from the *trans*-Golgi network to endosomes, we evaluated their role in APP metabolism and cell viability using mouse fibroblast MS cells deficient in the murine IGF-II receptor and corresponding MS9II cells overexpressing the human IGF-II receptors. Our results show that IGF-II receptor overexpression increases the protein levels of APP. This is accompanied by an increase of β-site APP-cleaving enzyme 1 levels and an increase of β- and γ-secretase enzyme activities, leading to enhanced Aβ production. At the cellular level, IGF-II receptor overexpression causes localization of APP in perinuclear tubular structures, an increase of lipid raft components, and increased lipid raft partitioning of APP. Finally, MS9II cells are more susceptible to staurosporine-induced cytotoxicity, which can be attenuated by β-secretase inhibitor. Together, these results highlight the potential contribution of IGF-II receptor to AD pathology not only by regulating expression/processing of APP but also by its role in cellular vulnerability.

3.2355 Ablation of retinal ciliopathy protein RPGR results in altered photoreceptor ciliary composition

Rao, K.N., Li, L., Anand, M. and Khanna, H.

*Scientific reports, **5**:11137 (2015)*

Cilia regulate several developmental and homeostatic pathways that are critical to survival. Sensory cilia of photoreceptors regulate phototransduction cascade for visual processing. Mutations in the ciliary protein RPGR (retinitis pigmentosa GTPase regulator) are a prominent cause of severe blindness disorders due to degeneration of mature photoreceptors. However, precise function of RPGR is still unclear. Here we studied the involvement of RPGR in ciliary trafficking by analyzing the composition of photoreceptor sensory cilia (PSC) in $Rpgr^{ko}$ retina. Using tandem mass spectrometry analysis followed by immunoblotting, we detected few alterations in levels of proteins involved in proteasomal function and vesicular trafficking in $Rpgr^{ko}$ PSC, prior to onset of degeneration. We also found alterations in the levels of high molecular weight soluble proteins in $Rpgr^{ko}$ PSC. Our data indicate RPGR regulates entry or retention of soluble proteins in photoreceptor cilia but spares the trafficking of key structural and phototransduction-associated proteins. Given a frequent occurrence of *RPGR* mutations in severe photoreceptor degeneration due to ciliary disorders, our results provide insights into pathways resulting in altered mature cilia function in ciliopathies.

3.2356 Mitochondrial m-calpain opens the mitochondrial permeability transition pore in ischemia–reperfusion

Shintani-Ishida, K. and Yoshida, K-i.

Int. J. Cardiol., ***197***, *26-32 (2015)*

Background/objectives
: Opening of the mitochondrial permeability transition pore (mPTP) is involved in ischemia–reperfusion injury. Isoforms of Ca^{2+}-activated cysteine proteases, calpains, are implicated in the development of myocardial infarction in ischemia–reperfusion. Growing evidence has revealed the presence of calpains in the mitochondria. We aimed to characterize mitochondrial calpains in the rat heart and to investigate the roles of calpains in mPTP opening after ischemia–reperfusion.
Methods and results
: Western blotting analysis showed the expression of μ-calpain, m-calpain and calpain 10 in mitochondria isolated from male Sprague-Dawley rats, but casein zymography detected only m-calpain activity. Subcellular fractionation of mitochondria demonstrated the distribution of m-calpain to the matrix fraction.

Addition of > 500 μM of Ca^{2+} to isolated mitochondria induced mitochondrial swelling, reflecting mPTP opening, and calpain activation. Ca^{2+}-induced mitochondrial swelling was inhibited partially by the calpain inhibitor calpeptin. These results support a partial contribution of calpain in the opening of the mPTP. The addition of Ca^{2+} to the mitochondria induced inactivation of complex I of the electron transport chain, and cleavage of the ND6 complex I subunit, which were inhibited by calpeptin. Mitochondria isolated from rat hearts that underwent 30 min of coronary occlusion followed by 30 min of reperfusion showed activation of mitochondrial calpains, ND6 cleavage, complex I inactivation, and mPTP opening, which were inhibited by pretreatment with calpain inhibitor 1.
Conclusions
: We demonstrated for the first time the presence of mitochondrial matrix m-calpain, and its contribution to complex I inactivation and mPTP opening after postischemic reperfusion in the rat heart.

3.2357 Cellular Uptake Mechanism of TCTP-PTD in Human Lung Carcinoma Cells
Kim, H.Y., Kim, S., Pyun, H.J., Maeng, J. and Lee, K.
*Mol. Pharmaceutics, **12(1)**, 194-203 (2015)*

We reported previously that human translationally controlled tumor protein (TCTP) contains, at its NH_2-terminus, a protein transduction domain (PTD), which we called TCTP-PTD, with the amino acid sequence MIIYRDLISH. In this report we describe how TCTP-PTD penetrates A549 human lung cancer cell membranes and promotes protein internalization. Cellular uptake of fluorescent TCTP-PTD and a recombinant fusion protein consisting of TCTP-PTD and GFP (green fluorescent protein) was analyzed by confocal fluorescence microscopy and flow cytometry. Inhibitor assays using several agents that perturb the internalization process revealed that TCTP-PTD transduces the cells partly via lipid-raft/caveola-dependent endocytosis and partly by macropinocytosis in a dynamin/actin/microtubule-dependent pathway. To trace the pathway followed by the penetration of TCTP-PTD, the localization of PTDs was investigated in the lipid-raft, subcellular, and ER fractions. We found that, after entry, TCTP-PTD is localized in the cytoplasm and cytoskeleton, but not in the nucleus, and is transported into endoplasmic reticulum (ER). Expression levels of caveolin-1 in A549 and HeLa cells are different, and these differences appear to contribute to the sensitivity of TCTP-PTD uptake inhibition, against lipid-raft depleter, nystatin. This elucidation of the underlying mechanism of TCTP-PTD translocation may help the design of approaches that employ TCTP-PTD in the cellular delivery of bioactive molecules.

3.2358 Bacterial Protoplast-Derived Nanovesicles as Vaccine Delivery System against Bacterial Infection
Kim, O.Y., Choi, S.J., jang, S.C., park, K-S., Kim, S.R., Choi, J.P., Lim, J.H., Lee, S-W., park, J., Di Vizio, D., Lötvall, J., Kim, Y-K. and Gho, Y.S.
*Nano Lett., **15(1)**, 266-274 (2015)*

The notion that widespread infectious diseases could be best managed by developing potent, adjuvant-free vaccines has resulted in the use of various biological immune-stimulating components as new vaccine candidates. Recently, extracellular vesicles, also known as exosomes and microvesicles in mammalian cells and outer membrane vesicles in Gram-negative bacteria, have gained attention for the next generation vaccine. However, the more invasive and effective the vaccine is in delivery, the more risk it holds for severe immune toxicity. Here, in optimizing the current vaccine delivery system, we designed bacterial protoplast-derived nanovesicles (PDNVs), depleted of toxic outer membrane components to generate a universal adjuvant-free vaccine delivery system. These PDNVs exhibited significantly higher productivity and safety than the currently used vaccine delivery vehicles and induced strong antigen-specific humoral and cellular immune responses. Moreover, immunization with PDNVs loaded with bacterial antigens conferred effective protection against bacterial sepsis in mice. These nonliving nanovesicles derived from bacterial protoplast open up a new avenue for the creation of next generation, adjuvant-free, less toxic vaccines to be used to prevent infectious diseases.

3.2359 Circulating microRNAs: emerging biomarkers for diagnosis and prognosis in patient with gastrointestinal cancers
Lindner, K., haier, J., Wang, Z., Watson, D.I., Hussey, D.J. and Hummel, R.
*Clinical Science, **128**, 1-15 (2015)*

To identify novel non-invasive biomarkers for improved detection, risk assessment and prognostic evaluation of cancer, expression profiles of circulating microRNAs are currently under evaluation. Circulating microRNAs are highly promising candidates in this context, as they present some key characteristics for cancer biomarkers: they are tissue-specific with reproducible expression and consistency

among individuals from the same species, they are potentially derived directly from the tumour and therefore might correlate with tumour progression and recurrence, and they are bound to proteins or contained in subcellular particles, such as microvesicles or exosomes, making them highly stable and resistant to degradation. The present review highlights the origin of circulating microRNAs, their stability in blood samples, and techniques to isolate exosomal microRNAs, and then addresses the current evidence supporting potential clinical applications of circulating miRNAs for diagnostic and prognostic purposes.

3.2360 ANKS1B Gene Product AIDA-1 Controls Hippocampal Synaptic Transmission by Regulating GluN2B Subunit Localization

Tindi, J.O., Chavez, A.E., Cvejic, S., Calvo-Ochoa, E., Castillo, P.E. and Jordan, B.A.
J. Neurosci., ***35(24)***, *8986-8996 (2015)*

NMDA receptors (NMDARs) are key mediators of glutamatergic transmission and synaptic plasticity, and their dysregulation has been linked to diverse neuropsychiatric and neurodegenerative disorders. While normal NMDAR function requires regulated expression and trafficking of its different subunits, the molecular mechanisms underlying these processes are not fully understood. Here we report that the amyloid precursor protein intracellular domain associated-1 protein (AIDA-1), which associates with NMDARs and is encoded by *ANKS1B*, a gene recently linked to schizophrenia, regulates synaptic NMDAR subunit composition. Forebrain-specific AIDA-1 conditional knock-out (cKO) mice exhibit reduced GluN2B-mediated and increased GluN2A-mediated synaptic transmission, and biochemical analyses show AIDA-1 cKO mice have low GluN2B and high GluN2A protein levels at isolated hippocampal synaptic junctions compared with controls. These results are corroborated by immunocytochemical and electrophysiological analyses in primary neuronal cultures following acute lentiviral shRNA-mediated knockdown of AIDA-1. Moreover, hippocampal NMDAR-dependent but not metabotropic glutamate receptor-dependent plasticity is impaired in AIDA-1 cKO mice, further supporting a role for AIDA-1 in synaptic NMDAR function. We also demonstrate that AIDA-1 preferentially associates with GluN2B and with the adaptor protein Ca^{2+}/calmodulin-dependent serine protein kinase and kinesin KIF17, which regulate the transport of GluN2B-containing NMDARs from the endoplasmic reticulum (ER) to synapses. Consistent with this function, GluN2B accumulates in ER-enriched fractions in AIDA-1 cKO mice. These findings suggest that AIDA-1 regulates NMDAR subunit composition at synapses by facilitating transport of GluN2B from the ER to synapses, which is critical for NMDAR plasticity. Our work provides an explanation for how AIDA-1 dysfunction might contribute to neuropsychiatric conditions, such as schizophrenia.

3.2361 Mass-Spectrometry-Based Molecular Characterization of Extracellular Vesicles: Lipidomics and Proteomics

Kreimer, S., Belov, A.M., Ghiran, I., Murthy, S.K., Frank, D.A. and Ivanov, A.R.
J. Proteome Res., ***14(6)***, *2367-2384 (2015)*

This review discusses extracellular vesicles (EVs), which are submicron-scale, anuclear, phospholipid bilayer membrane enclosed vesicles that contain lipids, metabolites, proteins, and RNA (micro and messenger). They are shed from many, if not all, cell types and are present in biological fluids and conditioned cell culture media. The term EV, as coined by the International Society of Extracellular Vesicles (ISEV), encompasses exosomes (30–100 nm in diameter), microparticles (100–1000 nm), apoptotic blebs, and other EV subsets. EVs have been implicated in cell–cell communication, coagulation, inflammation, immune response modulation, and disease progression. Multiple studies report that EV secretion from disease-affected cells contributes to disease progression, e.g., tumor niche formation and cancer metastasis. EVs are attractive sources of biomarkers due to their biological relevance and relatively noninvasive accessibility from a range of physiological fluids. This review is focused on the molecular profiling of the protein and lipid constituents of EVs, with emphasis on mass-spectrometry-based “omic” analytical techniques. The challenges in the purification and molecular characterization of EVs, including contamination of isolates and limitations in sample quantities, are discussed along with possible solutions. Finally, the review discusses the limited but growing investigation of post-translational modifications of EV proteins and potential strategies for future in-depth molecular characterization of EVs.

3.2362 Exosomes released by keratinocytes modulate melanocyte pigmentation

Lo Cicero, A., Delevoye, C., Gilles-Marsens, F., Loew, D., Dingli, F., Guere, C., Andre, N., Vie, K., van Niel, G. and Raposo, G.
Nature Communications, ***6****:7506 (2015)*

Cells secrete extracellular vesicles (EVs), exosomes and microvesicles, which transfer proteins, lipids and RNAs to regulate recipient cell functions. Skin pigmentation relies on a tight dialogue between keratinocytes and melanocytes in the epidermis. Here we report that exosomes secreted by keratinocytes enhance melanin synthesis by increasing both the expression and activity of melanosomal proteins. Furthermore, we show that the function of keratinocyte-derived exosomes is phototype-dependent and is modulated by ultraviolet B. In sum, this study uncovers an important physiological function for exosomes in human pigmentation and opens new avenues in our understanding of how pigmentation is regulated by intercellular communication in both healthy and diseased states.

3.2363 Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study
Zaborowski, M.P., Balaj, L., Breakfield, X.O. and Lai, C.P.
*BioScience, **65(8)**, 783-797 (2015)*

The release of extracellular vesicles (EVs), including exosomes and microvesicles, is a phenomenon shared by many cell types as a means of communicating with other cells and also potentially removing cell contents. The cargo of EVs includes the proteins, lipids, nucleic acids, and membrane receptors of the cells from which they originate. EVs released into the extracellular space can enter body fluids and potentially reach distant tissues. Once taken up by neighboring and/or distal cells, EVs can transfer functional cargo that may alter the status of recipient cells, thereby contributing to both physiological and pathological processes. In this article, we will focus on EV composition, mechanisms of uptake, and their biological effects on recipient cells. We will also discuss established and recently developed methods used to study EVs, including isolation, quantification, labeling and imaging protocols, as well as RNA analysis.

3.2364 A novel chimeric aequorin fused with caveolin-1 reveals a sphingosine kinase 1-regulated Ca2 + microdomain in the caveolar compartment
Pulli, I., Blom, T., Löf, C., mafnusson, M., Rimessi, A., pinton, P. and Törnquist, K.
*Biochim. Biophys. Acta, **1853**, 2173-2182 (2015)*

Caveolae are plasma membrane invaginations enriched in sterols and sphingolipids. Sphingosine kinase 1 (SK1) is an oncogenic protein that converts sphingosine to sphingosine 1-phosphate (S1P), which is a messenger molecule involved in calcium signaling. Caveolae contain calcium responsive proteins, but the effects of SK1 or S1P on caveolar calcium signaling have not been investigated. We generated a Caveolin-1–Aequorin fusion protein (Cav1–Aeq) that can be employed for monitoring the local calcium concentration at the caveolae ($[Ca^{2+}]$cav). In HeLa cells, Cav1–Aeq reported different $[Ca^{2+}]$ as compared to the plasma membrane $[Ca^{2+}]$ in general (reported by SNAP25–Aeq) or as compared to the cytosolic $[Ca^{2+}]$ (reported by cyt-Aeq). The Ca^{2+} signals detected by Cav1–Aeq were significantly attenuated when the caveolar structures were disrupted by methyl-β-cyclodextrin, suggesting that the caveolae are specific targets for Ca^{2+} signaling. HeLa cells overexpressing SK1 showed increased $[Ca^{2+}]$cav during histamine-induced Ca^{2+} mobilization in the absence of extracellular Ca^{2+} as well as during receptor-operated Ca^{2+} entry (ROCE). The SK1-induced increase in $[Ca^{2+}]$cav during ROCE was reverted by S1P receptor antagonists. In accordance, pharmacologic inhibition of SK1 reduced the $[Ca^{2+}]$cav during ROCE. S1P treatment stimulated the $[Ca^{2+}]$cav upon ROCE. The Ca^{2+} responses at the plasma membrane in general were not affected by SK1 expression. In summary, our results show that SK1/S1P-signaling regulates Ca^{2+} signals at the caveolae. This article is part of a Special Issue entitled: 13th European Symposium on Calcium.

3.2365 A method to investigate protein association with intact sealed mycobacterial membrane vesicles
D'Lima, N.G. and Teschke, C.M.
*Anal. Biochem., **485**, 109-111 (2015)*

In mycobacteria, probing the association of cytoplasmic proteins with the membrane itself, as well as with integral or peripheral membrane proteins, is limited by the difficulty in extracting intact sealed membrane vesicles due to the complex cell wall structure. Here we tested the association of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins with intact membrane vesicles by a flotation assay using iodixanol density gradients. These protocols have wide applications for studying the association of other mycobacterial cytoplasmic proteins with the membrane and membrane-associated proteins.

3.2366 Klebsiella pneumoniae O antigen loss alters the outer membrane protein composition and the selective packaging of proteins into secreted outer membrane vesicles
Cahill, B.K., seeley, K.W., Gutel, D. and Ellis, T.N.

Microbiol Res., ***180***, *1-10 (2015)*

Klebsiella pneumoniae is a nosocomial pathogen which naturally secretes lipopolysaccharide (LPS) and cell envelope associated proteins into the environment through the production of outer membrane vesicles (OMVs). The loss of the LPS O antigen has been demonstrated in other bacterial species to significantly alter the composition of OMVs. Therefore, this study aimed to comprehensively analyze the impact of O antigen loss on the sub-proteomes of both the outer membrane and secreted OMVs from *K. pneumoniae*. As determined by LC–MS/MS, OMVs were highly enriched with outer membrane proteins involved in cell wall, membrane, and envelope biogenesis as compared to the source cellular outer membrane. Deletion of *wbbO*, the enzyme responsible for O antigen attachment to LPS, decreased but did not eliminate this enrichment effect. Additionally, loss of O antigen resulted in OMVs with increased numbers of proteins involved in post-translational modification, protein turnover, and chaperones as compared to secreted vesicles from the wild type. This alteration of OMV composition may be a compensatory mechanism to deal with envelope stress. This comprehensive analysis confirms the highly distinct protein composition of OMVs as compared to their source membrane, and provides evidence for a selective sorting mechanism that involves LPS polysaccharides. These data support the hypothesis that modifications to LPS alters both the mechanics of protein sorting and the contents of secreted OMVs and significantly impacts the protein composition of the outer membrane.

3.2367 Calcium release through P2X4 activates calmodulin to promote endolysosomal membrane fusion
Cao, Q., Zhang, X.Z., Zou, Y., Murrell-Lagnado, R., Zhu, M.X. and Dong, X-P.
J. Cell Biol., ***209(6)***, *879-894 (2015)*

Intra-endolysosomal Ca^{2+} release is required for endolysosomal membrane fusion with intracellular organelles. However, the molecular mechanisms for intra-endolysosomal Ca^{2+} release and the downstream Ca^{2+} targets involved in the fusion remain elusive. Previously, we demonstrated that endolysosomal P2X4 forms channels activated by luminal adenosine triphosphate in a pH-dependent manner. In this paper, we show that overexpression of P2X4, as well as increasing endolysosomal P2X4 activity by alkalinization of endolysosome lumen, promoted vacuole enlargement in cells and endolysosome fusion in a cell-free assay. These effects were prevented by inhibiting P2X4, expressing a dominant-negative P2X4 mutant, and disrupting the P2X4 gene. We further show that P2X4 and calmodulin (CaM) form a complex at endolysosomal membrane where P2X4 activation recruits CaM to promote fusion and vacuolation in a Ca^{2+}-dependent fashion. Moreover, P2X4 activation-triggered fusion and vacuolation were suppressed by inhibiting CaM. Our data thus suggest a new molecular mechanism for endolysosomal membrane fusion involving P2X4-mediated endolysosomal Ca^{2+} release and subsequent CaM activation.

3.2368 CIN85 modulates TGFβ signaling by promoting the presentation of TGFβ receptors on the cell surface
Yakymovych, I., Yakymovych, M., Zang, G., Mu, Y., Bergh, A., Landström, M. and Heldin, C-H.
J. Cell Biol., ***210(2)***, *319-332 (2015)*

Members of the transforming growth factor β (TGFβ) family initiate cellular responses by binding to TGFβ receptor type II (TβRII) and type I (TβRI) serine/threonine kinases, whereby Smad2 and Smad3 are phosphorylated and activated, promoting their association with Smad4. We report here that TβRI interacts with the SH3 domains of the adaptor protein CIN85 in response to TGFβ stimulation in a TRAF6-dependent manner. Small interfering RNA–mediated knockdown of CIN85 resulted in accumulation of TβRI in intracellular compartments and diminished TGFβ-stimulated Smad2 phosphorylation. Overexpression of CIN85 instead increased the amount of TβRI at the cell surface. This effect was inhibited by a dominant-negative mutant of Rab11, suggesting that CIN85 promoted recycling of TGFβ receptors. CIN85 enhanced TGFβ-stimulated Smad2 phosphorylation, transcriptional responses, and cell migration. CIN85 expression correlated with the degree of malignancy of prostate cancers. Collectively, our results reveal that CIN85 promotes recycling of TGFβ receptors and thereby positively regulates TGFβ signaling.

3.2369 Ubiquitin-Mediated Proteasomal Degradation of Oleosins is Involved in Oil Body Mobilization During Post-Germinative Seedling Growth in Arabidopsis
Deruyffelaere, C., Bouchez, I., Morin, H., Guillot, A., Miquel, M., Froissard, M., Chardot, st. and D'Andrea, S.
Plant Cell Physiol., ***56(7)***, *1374-1387 (2015)*

In oleaginous seeds, lipids—stored in organelles called oil bodies (OBs)—are degraded post-germinatively to provide carbon and energy for seedling growth. To date, little is known about how OB coat proteins, known as oleosins, control OB dynamics during seed germination. Here, we demonstrated that the sequential proteolysis of the five *Arabidopsis thaliana* oleosins OLE1–OLE5 begins just prior to lipid degradation. Several post-translational modifications (e.g. phosphorylation and ubiquination) of oleosins were concomitant with oleosin degradation. Phosphorylation occurred only on the minor OLE5 and on an 8 kDa proteolytic fragment of OLE2. A combination of immunochemical and proteomic approaches revealed ubiquitination of the four oleosins OLE1–OLE4 at the onset of OB mobilization. Ubiquitination topology was surprisingly complex. OLE1 and OLE2 were modified by three distinct and predominantly exclusive motifs: monoubiquitin, K48-linked diubiquitin ($K48Ub_2$) and K63-linked diubiquitin. Ubiquitinated oleosins may be channeled towards specific degradation pathways according to ubiquitination type. One of these pathways was identified as the ubiquitin–proteasome pathway. A proteasome inhibitor (MG132) reduced oleosin degradation and induced cytosolic accumulation of $K48Ub_2$–oleosin aggregates. These results indicate that $K48Ub_2$-modified oleosins are selectively extracted from OB coat and degraded by the proteasome. Proteasome inhibition also reduced lipid hydrolysis, providing in vivo evidence that oleosin degradation is required for lipid mobilization.

3.2370 X chromosome-linked intellectual disability protein PQBP1 associates with and regulates the translation of specific mRNAs
Wan, D., Zhang, Z.C., Zhang, X., Li, Q. and Han, J.
Hum. Mol. Genet., ***24(16)***, *4599-4614 (2015)*

X chromosome-linked intellectual disability is a common developmental disorder, and mutations of the polyglutamine-binding protein 1 (PQBP1) gene have been linked to this disease. In addition to existing in the nucleus as a splicing factor, PQBP1 is also found in cytoplasmic RNA granules, where it associates with RNA-binding proteins. However, the roles of cytoplasmic PQBP1 are largely unknown. Here, we show that the *Drosophila* homolog of PQBP1 (dPQBP1) is present in the cytoplasm of photoreceptor cells, and its loss results in defective rhabdomere morphogenesis, which is due to impaired Chaoptin translation. We also show that dPQBP1 regulates mRNA translation by interacting with dFMR1, which binds to specific mRNAs and facilitates their assembly into translating ribosomes, a function that is conserved for human PQBP1 and FMRP. Our findings reveal the conserved function of PQBP1 in mRNA translation and provide molecular insights into the pathogenic mechanisms underlying Renpenning syndrome.

3.2371 Intracellular Catabolism of an Antibody Drug Conjugate with a Noncleavable Linker
Rock, B.M., Tometsko, M.E., patel, S.K., Hamblett, K.J., Fanslow, W. and Rock, D.A.
Drug Metab. Dispos., ***43***, *1341-1344 (2015)*

Antibody drug conjugates are emerging as a powerful class of antitumor agents with efficacy across a range of cancers; therefore, understanding the disposition of this class of therapeutic is crucial. Reported here is a method of enriching a specific organelle (lysosome) to understand the catabolism of an anti-CD70 Ab-MCC-DM1, an antibody drug conjugate with a noncleavable linker. With such techniques a higher degree of concentration-activity relationship can be established for in vitro cell lines; this can aid in understanding the resultant catabolite concentrations necessary to exert activity.

3.2372 Platelet microparticles are internalized in neutrophils via the concerted activity of 12-lipoxygenase and secreted phospholipase A2-IIA
Duchez, A-C. et al
PNAS, ***112(27)***, *E3654-E3573 (2015)*

Platelets are anucleated blood elements highly potent at generating extracellular vesicles (EVs) called microparticles (MPs). Whereas EVs are accepted as an important means of intercellular communication, the mechanisms underlying platelet MP internalization in recipient cells are poorly understood. Our lipidomic analyses identified 12(S)-hydroxyeicosatetranoic acid [12(S)-HETE] as the predominant eicosanoid generated by MPs. Mechanistically, 12(S)-HETE is produced through the concerted activity of secreted phospholipase A_2 IIA ($sPLA_2$-IIA), present in inflammatory fluids, and platelet-type 12-lipoxygenase (12-LO), expressed by platelet MPs. Platelet MPs convey an elaborate set of transcription factors and nucleic acids, and contain mitochondria. We observed that MPs and their cargo are internalized by activated neutrophils in the endomembrane system via 12(S)-HETE. Platelet MPs are found inside neutrophils isolated from the joints of arthritic patients, and are found in neutrophils only in the presence of $sPLA_2$-IIA and 12-LO in an in vivo model of autoimmune inflammatory arthritis. Using a combination

of genetically modified mice, we show that the coordinated action of $sPLA_2$-IIA and 12-LO promotes inflammatory arthritis. These findings identify 12(S)-HETE as a trigger of platelet MP internalization by neutrophils, a mechanism highly relevant to inflammatory processes. Because $sPLA_2$-IIA is induced during inflammation, and 12-LO expression is restricted mainly to platelets, these observations demonstrate that platelet MPs promote their internalization in recipient cells through highly regulated mechanisms.

3.2373 O-glycans direct selectin ligands to lipid rafts on leukocytes
Shao, B., Yago, T., Setiadi, H., Wang, Y., Mehta-D'souza, P., Fu, J., Crocker, P.R., Rodgers, W., Xia, L and McEver, R.P.
*PNAS, **112(28)**, 8661-8666 (2015)*

Palmitoylated cysteines typically target transmembrane proteins to domains enriched in cholesterol and sphingolipids (lipid rafts). P-selectin glycoprotein ligand-1 (PSGL-1), CD43, and CD44 are O-glycosylated proteins on leukocytes that associate with lipid rafts. During inflammation, they transduce signals by engaging selectins as leukocytes roll in venules, and they move to the raft-enriched uropods of polarized cells upon chemokine stimulation. It is not known how these glycoproteins associate with lipid rafts or whether this association is required for signaling or for translocation to uropods. Here, we found that loss of core 1-derived O-glycans in murine *C1galt1*$^{-/-}$ neutrophils blocked raft targeting of PSGL-1, CD43, and CD44, but not of other glycosylated proteins, as measured by resistance to solubilization in nonionic detergent and by copatching with a raft-resident sphingolipid on intact cells. Neuraminidase removal of sialic acids from wild-type neutrophils also blocked raft targeting. *C1galt1*$^{-/-}$ neutrophils or neuraminidase-treated neutrophils failed to activate tyrosine kinases when plated on immobilized anti–PSGL-1 or anti-CD44 $F(ab')_2$. Furthermore, *C1galt1*$^{-/-}$ neutrophils incubated with anti–PSGL-1 $F(ab')_2$ did not generate microparticles. In marked contrast, PSGL-1, CD43, and CD44 moved normally to the uropods of chemokine-stimulated *C1galt1*$^{-/-}$ neutrophils. These data define a role for core 1-derived O-glycans and terminal sialic acids in targeting glycoprotein ligands for selectins to lipid rafts of leukocytes. Preassociation of these glycoproteins with rafts is required for signaling but not for movement to uropods.

3.2374 Cysteine cathepsins are essential in lysosomal degradation of α-synuclein
McGlinchey, R.P. and Lee, J.C.
*PNAS, **112(30)**, 9322-9327 (2015)*

A cellular feature of Parkinson's disease is cytosolic accumulation and amyloid formation of α-synuclein (α-syn), implicating a misregulation or impairment of protein degradation pathways involving the proteasome and lysosome. Within lysosomes, cathepsin D (CtsD), an aspartyl protease, is suggested to be the main protease for α-syn clearance; however, the protease alone only generates amyloidogenic C terminal-truncated species (e.g., 1–94, 5–94), implying that other proteases and/or environmental factors are needed to facilitate degradation and to avoid α-syn aggregation in vivo. Using liquid chromatography–mass spectrometry, to our knowledge, we report the first peptide cleavage map of the lysosomal degradation process of α-syn. Studies of purified mouse brain and liver lysosomal extracts and individual human cathepsins demonstrate a direct involvement of cysteine cathepsin B (CtsB) and L (CtsL). Both CtsB and CtsL cleave α-syn within its amyloid region and circumvent fibril formation. For CtsD, only in the presence of anionic phospholipids can this protease cleave throughout the α-syn sequence, suggesting that phospholipids are crucial for its activity. Taken together, an interplay exists between α-syn conformation and cathepsin activity with CtsL as the most efficient under the conditions examined. Notably, we discovered that CtsL efficiently degrades α-syn amyloid fibrils, which by definition are resistant to broad spectrum proteases. This work implicates CtsB and CtsL as essential in α-syn lysosomal degradation, establishing groundwork to explore mechanisms to enhance their cellular activity and levels as a potential strategy for clearance of α-syn.

3.2375 Calcium-dependent membrane association of a flagellar calcium sensor does not require calcium binding
Maric, D., Olson, C.L., Xu, X., Ames, J.B. and Engman, D.M.
*Mol. Biochem. Parasitol., **201(1)**, 72-75 (2015)*

Flagellar calcium-binding protein (FCaBP) is a dually acylated Ca^{2+} sensor in the *Trypanosoma cruzi* flagellar membrane that undergoes a massive conformational change upon Ca^{2+} binding. It is similar to neuronal Ca^{2+} sensors, like recoverin, which regulate their binding partners through a calcium acyl switch mechanism. FCaBP is washed out of permeabilized cells with buffers containing EDTA, indicating Ca^{2+}-dependent flagellar membrane association. We hypothesized that, like recoverin, FCaBP projects its acyl

groups in the presence of Ca^{2+}, permitting flagellar membrane and binding partner association and that it sequesters the acyl groups in low Ca^{2+}, disassociating from the membrane and releasing its binding partner to perform a presumed enzymatic function. The X-ray crystal structure of FCaBP suggests that the acyl groups are always exposed, so we set out to test our hypothesis directly. We generated *T. cruzi* transfectants expressing FCaBP or Ca^{2+}-binding mutant $FCaBP^{E151Q/E188Q}$ and recombinant wildtype and mutant proteins as well. Both FCaBP and $FCaBP^{E151Q/E188Q}$ were found to associate with lipid rafts, indicating the Ca^{2+}-independence of this association. To our initial surprise, $FCaBP^{E151Q/E188Q}$, like wildtype FCaBP, exhibited Ca^{2+}-dependent flagellar membrane association, even though this protein does not bind Ca^{2+} itself [16]. One possible explanation for this is that $FCaBP^{E151Q/E188Q}$, like some other Ca^{2+} sensors, may form dimers and that dimerization of $FCaBP^{E151Q/E188Q}$ with endogenous wildtype FCaBP might explain its Ca^{2+}-dependent localization. Indeed both proteins are able to form dimers in the presence and absence of Ca^{2+}. These results suggest that FCaBP possesses two distinct Ca^{2+}-dependent interactions—one involving a Ca^{2+}-induced change in conformation and another perhaps involving binding partner association.

3.2376 Virulence from vesicles: Novel mechanisms of host cell injury by Escherichia coli O104:H4 outbreak strain

Kunsmann, L., Rüter, C., Bauwens, A., Greune, L., Glüder, M., Kemper, B., Fruth, A., Wai, S.N., He, X., Lloubes, R., Schmidt, M.A., Dobrindt, U., Mellmann, A., Karch, H. and Bielaszewska, M.
*Scientific Reports, **5**: 13253 (2015)*

The highly virulent Escherichia coli O104:H4 that caused the large 2011 outbreak of diarrhoea and haemolytic uraemic syndrome secretes blended virulence factors of enterohaemorrhagic and enteroaggregative E. coli, but their secretion pathways are unknown. We demonstrate that the outbreak strain releases a cocktail of virulence factors via outer membrane vesicles (OMVs) shed during growth. The OMVs contain Shiga toxin (Stx) 2a, the major virulence factor of the strain, Shigella enterotoxin 1, H4 flagellin, and O104 lipopolysaccharide. The OMVs bind to and are internalised by human intestinal epithelial cells via dynamin-dependent and Stx2a-independent endocytosis, deliver the OMV-associated virulence factors intracellularly and induce caspase-9-mediated apoptosis and interleukin-8 secretion. Stx2a is the key OMV component responsible for the cytotoxicity, whereas flagellin and lipopolysaccharide are the major interleukin-8 inducers. The OMVs represent novel ways for the E. coli O104:H4 outbreak strain to deliver pathogenic cargoes and injure host cells.

3.2377 Role for Rab10 in Methamphetamine-Induced Behavior

Vanderwerf, S.M., Buck, D.C., Wilmarth, P.A., Sears, L.M., David, L.L., Morton, D.B. and Neve, K.A.
*PloS One, **10(8)**, e0136167 (2015)*

Lipid rafts are specialized, cholesterol-rich membrane compartments that help to organize transmembrane signaling by restricting or promoting interactions with subsets of the cellular proteome. The hypothesis driving this study was that identifying proteins whose relative abundance in rafts is altered by the abused psychostimulant methamphetamine would contribute to fully describing the pathways involved in acute and chronic effects of the drug. Using a detergent-free method for preparing rafts from rat brain striatal membranes, we identified density gradient fractions enriched in the raft protein flotillin but deficient in calnexin and the transferrin receptor, markers of non-raft membranes. Dopamine D1- and D2-like receptor binding activity was highly enriched in the raft fractions, but pretreating rats with methamphetamine (2 mg/kg) once or repeatedly for 11 days did not alter the distribution of the receptors. LC-MS analysis of the protein composition of raft fractions from rats treated once with methamphetamine or saline identified methamphetamine-induced changes in the relative abundance of 23 raft proteins, including the monomeric GTP-binding protein Rab10, whose abundance in rafts was decreased 2.1-fold by acute methamphetamine treatment. Decreased raft localization was associated with a selective decrease in the abundance of Rab10 in a membrane fraction that includes synaptic vesicles and endosomes. Inhibiting Rab10 activity by pan-neuronal expression of a dominant-negative Rab10 mutant in *Drosophila melanogaster* decreased methamphetamine-induced activity and mortality and decreased caffeine-stimulated activity but not mortality, whereas inhibiting Rab10 activity selectively in cholinergic neurons had no effect. These results suggest that activation and redistribution of Rab10 is critical for some of the behavioral effects of psychostimulants.

3.2378 Lysosome-Related Effector Vesicles in T Lymphocytes and NK Cells

Lettau, M., kabelitz, D. and Janssen, O.
*Scand. J. Immunol., **82(3)**, 235-243 (2015)*

Lysosome-related secretory organelles combine metabolic functions of conventional lysosomes with an inducible secretory potential. Specialized variants of such bi-functional organelles are present in several haematopoietic cell types that store, mobilize and/or secrete effector proteins, for example in mast cells, macrophages or cytotoxic effector cells. In the case of T lymphocytes and NK cells, it was believed that secretory lysosomes serve as a common storage and transport compartment for the most relevant cytotoxic effector proteins including FasL, perforin, granzymes and granulysin. However, recent observations suggest that cytotoxic effector cells might be able to mobilize two distinct lysosomal entities in order to react to differential stimulation with either FasL surface appearance or degranulation-associated release of perforin and granzymes. This assumption is supported by the proteomic characterization of enriched organelles from T and NK cells. FasL-associated light lysosomes biochemically segregate from morphologically distinct heavy lysosomes that preferentially contain granzymes, perforin and mature granulysin. Here, we briefly summarize the current knowledge about cargo proteins that are stored and transported in secretory vesicles and how these vesicles might be generated and mobilized. In addition, we describe common features and major differences of the two distinct effector organelles and discuss how these observations might expand existing models of cytotoxic effector function.

3.2379 Enhancement of BACE1 Activity by p25/Cdk5-Mediated Phosphorylation in Alzheimer's Disease
Song, W-J., Son, M-Y., Lee, H-W., Seo, H., Kim, J.H. and Chung, S-H.
*PloS One, **10(8)**, e0136950 (2015)*

The activity of beta-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) is elevated during aging and in sporadic Alzheimer's disease (AD), but the underlying mechanisms of this change are not well understood. p25/Cyclin-dependent kinase 5 (Cdk5) has been implicated in the pathogenesis of several neurodegenerative diseases, including AD. Here, we describe a potential mechanism by which BACE activity is increased in AD brains. First, we show that BACE1 is phosphorylated by the p25/Cdk5 complex at Thr252 and that this phosphorylation increases BACE1 activity. Then, we demonstrate that the level of phospho-BACE1 is increased in the brains of AD patients and in mammalian cells and transgenic mice that overexpress p25. Furthermore, the fraction of p25 prepared from iodixanol gradient centrifugation was unexpectedly protected by protease digestion, suggesting that p25/Cdk5-mediated BACE1 phosphorylation may occur in the lumen. These results reveal a link between p25 and BACE1 in AD brains and suggest that upregulated Cdk5 activation by p25 accelerates AD pathogenesis by enhancing BACE1 activity via phosphorylation.

3.2380 Isolation of Peroxisomes from Yeast
Cramer, J., Effelsberg, D., Girzalsky, W. and Erdmann, R.
Cold Spring Harbor Protocols, pdb.top074500 (2015)

Peroxisomes are multifunctional, dynamic organelles present in nearly all eukaryotic cells. Determining their structural and functional characteristics often requires obtaining isolated and purified peroxisomes via subcellular fractionation. Subcellular fractionation techniques are generally based on a three-step procedure: preparation of a cell-free homogenate (postnuclear supernatant), generation of an organellar pellet by differential centrifugation, and density gradient centrifugation. Here we introduce methods for small-scale isolation of peroxisomes from yeast cells using different gradient media as well as large-scale purification using a two-step gradient centrifugation.

3.2381 Small-Scale Purification of Peroxisomes for Analytical Applications
Cramer, J., Effelsberg, D., Girzalsky, W. and Erdmann, R.
Cold Spring Harbor Protocols, pdb.prot083717 (2015)
This protocol describes the isolation of peroxisomes from *Saccharomyces cerevisiae* by density gradient centrifugation using a sucrose, OptiPrep, or OptiPrep/sucrose gradient. Oleic acid–induced cells are first converted to spheroplasts using lyticase for cell wall digestion. Spheroplasts are homogenized, and nuclei and cell debris are removed by low-speed centrifugation to produce a postnuclear supernatant (PNS). Separation of the PNS by density gradient centrifugation is suitable for many analytical applications; however, to increase the yield of peroxisomes, further fractionation of the PNS is possible. Differential centrifugation of the PNS allows removal of the cytosol and other contaminating organelles, resulting in an organellar pellet (OP) enriched in peroxisomes and mitochondria that can be loaded onto the density gradient. Following density gradient centrifugation of the PNS or OP, fractions are collected from the bottom of the centrifuge tube. The distribution of organelles, including peroxisome peak fractions, is characterized by measurement of marker enzyme activity.

3.2382 Amaranthus leucocarpus lectin recognizes a moesin-like O-glycoprotein and costimulates murine CD3-activated CD4+ T cells

Arenas-Del Angel, M., Legorreta-Herrera, M., Mendoza-Hernandez, G., garfias, Y., Chavez, R., Zenteno, E. and Lascurain, R.

*Immunity, Inflammation and Disease, **3(3)**, 182-195 (2015)*

The Galβ1,3GalNAcα1,O-Ser/Thr specific lectin from *Amaranthus leucocarpus* (*ALL*) binds a ~70 kDa glycoprotein on murine T cell surface. We show that in the absence of antigen presenting cells, murine $CD4^+$ T cells activated by an anti-CD3 antibody plus *ALL* enhanced cell proliferation similar to those cells activated via CD3/CD28 at 48 h of culture. Moreover, *ALL* induced the production of IL-4, IL-10, TNF-alpha, and TGF-beta in CD3-activated cells. Proteomic assay using two-dimensional electrophoresis and far-Western blotting, *ALL* recognized two prominent proteins associated to the lipid raft microdomains in CD3/CD28-activated $CD4^+$ T cells. By mass spectrometry, the peptide fragments from *ALL*-recognized proteins showed sequences with 33% homology to matricin (gi|347839 NCBInr) and 41% identity to an unnamed protein related to moesin (gi|74186081 NCBInr). Confocal microscopy analysis of CD3/CD28-activated $CD4^+$ T cells confirmed that staining by *ALL* colocalized with anti-moesin FERM domain antibody along the plasma membrane and in the intercellular contact sites. Our findings suggest that a moesin-like O-glycoprotein is the *ALL*-recognized molecule in lipid rats, which induces costimulatory signals on $CD4^+$ T cells.

3.2383 Exosomes released by chronic lymphocytic leukemia cells induce the transition of stromal cells into cancer-associated fibroblasts

Paggetti, J., Haderk, F., Seiffert, M., janji, B., Distler, U., Ammerlaan, W., Kim, Y.J., Adam, J., Lichter, P., Solary, E., Berchem, G. and Moussay, E.

*Blood, **126(9)**, 1106-1117 (2015)*

Exosomes derived from solid tumor cells are involved in immune suppression, angiogenesis, and metastasis, but the role of leukemia-derived exosomes has been less investigated. The pathogenesis of chronic lymphocytic leukemia (CLL) is stringently associated with a tumor-supportive microenvironment and a dysfunctional immune system. Here, we explore the role of CLL-derived exosomes in the cellular and molecular mechanisms by which malignant cells create this favorable surrounding. We show that CLL-derived exosomes are actively incorporated by endothelial and mesenchymal stem cells ex vivo and in vivo and that the transfer of exosomal protein and microRNA induces an inflammatory phenotype in the target cells, which resembles the phenotype of cancer-associated fibroblasts (CAFs). As a result, stromal cells show enhanced proliferation, migration, and secretion of inflammatory cytokines, contributing to a tumor-supportive microenvironment. Exosome uptake by endothelial cells increased angiogenesis ex vivo and in vivo, and coinjection of CLL-derived exosomes and CLL cells promoted tumor growth in immunodeficient mice. Finally, we detected α-smooth actin–positive stromal cells in lymph nodes of CLL patients. These findings demonstrate that CLL-derived exosomes actively promote disease progression by modulating several functions of surrounding stromal cells that acquire features of cancer-associated fibroblasts.

3.2384 The prion protein inhibits monocytic cell migration by stimulating β1 integrin adhesion and uropod formation

Richardson, D.D., Tol, S., Valle-Encinas, E., Pleguezuelos, C., Bierings, R., Geerts, D. and Fernandez-Borja, M.

*J. Cell Sci., **128**, 3018-3029 (2015)*

The broad tissue distribution and evolutionary conservation of the glycosylphosphatidylinositol (GPI)-anchored prion protein (PrP, also known as PRNP) suggests that it plays a role in cellular homeostasis. Given that integrin adhesion determines cell behavior, the proposed role of PrP in cell adhesion might underlie the various *in vitro* and *in vivo* effects associated with PrP loss-of-function, including the immune phenotypes described in $PrP^{-/-}$ mice. Here, we investigated the role of PrP in the adhesion and (transendothelial) migration of human (pro)monocytes. We found that PrP regulates β1-integrin-mediated adhesion of monocytes. Additionally, PrP controls the cell morphology and migratory behavior of monocytes: PrP-silenced cells show deficient uropod formation on immobilized VCAM and display bleb-like protrusions on the endothelium. Our data further show that PrP regulates ligand-induced integrin activation. Finally, we found that PrP controls the activation of several proteins involved in cell adhesion and migration, including RhoA and its effector cofilin, as well as proteins of the ERM family. We propose

that PrP modulates β1 integrin adhesion and migration of monocytes through RhoA-induced actin remodeling mediated by cofilin, and through the regulation of ERM-mediated membrane–cytoskeleton linkage.he prion protein inhibits

3.2385 Microtubule motors transport phagosomes in the RPE, and lack of KLC1 leads to AMD-like pathogenesis
Jiang, M., Easteve-Rudd, J., Lapes, V.S., Diemer, T., Lillo, C., Rump, A. and Williams, D.S.
J. Cell Biol., ***210(4)***, *595-611 (2015)*

The degradation of phagosomes, derived from the ingestion of photoreceptor outer segment (POS) disk membranes, is a major role of the retinal pigment epithelium (RPE). Here, POS phagosomes were observed to associate with myosin-7a, and then kinesin-1, as they moved from the apical region of the RPE. Live-cell imaging showed that the phagosomes moved bidirectionally along microtubules in RPE cells, with kinesin-1 light chain 1 (KLC1) remaining associated in both directions and during pauses. Lack of KLC1 did not inhibit phagosome speed, but run length was decreased, and phagosome localization and degradation were impaired. In old mice, lack of KLC1 resulted in RPE pathogenesis that was strikingly comparable to aspects of age-related macular degeneration (AMD), with an excessive accumulation of RPE and sub-RPE deposits, as well as oxidative and inflammatory stress responses. These results elucidate mechanisms of POS phagosome transport in relation to degradation, and demonstrate that defective microtubule motor transport in the RPE leads to phenotypes associated with AMD.

3.2386 Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome
Linder, B., Groszhik, A.V., Olarerin-George, A.O., Meydan, C., Mason, C.E. and Jaffrey, S.R.
Nature Methods, ***12(8)***, *767-772 (2015)*

N^6-methyladenosine (m6A) is the most abundant modified base in eukaryotic mRNA and has been linked to diverse effects on mRNA fate. Current mapping approaches localize m6A residues to transcript regions 100–200 nt long but cannot identify precise m6A positions on a transcriptome-wide level. Here we developed m6A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP) and used it to demonstrate that antibodies to m6A can induce specific mutational signatures at m6A residues after ultraviolet light–induced antibody-RNA cross-linking and reverse transcription. We found that these antibodies similarly induced mutational signatures at N^6,2′-*O*-dimethyladenosine (m6Am), a modification found at the first nucleotide of certain mRNAs. Using these signatures, we mapped m6A and m6Am at single-nucleotide resolution in human and mouse mRNA and identified small nucleolar RNAs (snoRNAs) as a new class of m6A-containing non-coding RNAs (ncRNAs).

3.2387 Neurofilament subunits are integral components of synapses and modulate neurotransmission and behavior in vivo
Yuan, A., Sershen, H., Veeranna, Basavarajappa, B.S., Kumar, A., Hashim, A., Berg, M., Lee, J-H., Sato, Y., Rao, M.V., Mohan, P.S., Dyakin, V., Julien, J-P., Lee, VM-Y. and Nixon, R.A.
Molecular Psychiatry, ***20(8)***, *986-994 (2015)*

Synaptic roles for neurofilament (NF) proteins have rarely been considered. Here, we establish all four NF subunits as integral resident proteins of synapses. Compared with the population in axons, NF subunits isolated from synapses have distinctive stoichiometry and phosphorylation state, and respond differently to perturbations *in vivo*. Completely eliminating NF proteins from brain by genetically deleting three subunits (α-internexin, NFH and NFL) markedly depresses hippocampal long-term potentiation induction without detectably altering synapse morphology. Deletion of NFM in mice, but not the deletion of any other NF subunit, amplifies dopamine D1-receptor-mediated motor responses to cocaine while redistributing postsynaptic D1-receptors from endosomes to plasma membrane, consistent with a specific modulatory role of NFM in D1-receptor recycling. These results identify a distinct pool of synaptic NF subunits and establish their key role in neurotransmission *in vivo*, suggesting potential novel influences of NF proteins in psychiatric as well as neurological states.

3.2388 Identification of P-glycoprotein co-fractionating proteins and specific binding partners in rat brain microvessels
Tome, M.E., Schaefer, C.P., Jacobs, L.M., Zhang, Y., Herndon, J.M., Matty, F.O. and davis, T.P.
J. Neurochem., ***134(2)***, *200-210 (2015)*

Drug delivery to the brain for the treatment of pathologies with a CNS component is a significant clinical challenge. P-glycoprotein (PgP), a drug efflux pump in the endothelial cell membrane, is a major factor in preventing therapeutics from crossing the blood-brain barrier (BBB). Identifying PgP regulatory mechanisms is key to developing agents to modulate PgP activity. Previously, we found that PgP trafficking was altered concomitant with increased PgP activity and disassembly of high molecular weight PgP-containing complexes during acute peripheral inflammatory pain. These data suggest that PgP activity is post-translationally regulated at the BBB. The goal of the current study was to identify proteins that co-localize with PgP in rat brain microvessel endothelial cell membrane microdomains and use the data to suggest potential regulatory mechanisms. Using new density gradients of microvessel homogenates, we identified two unique pools (1,2) of PgP in membrane fractions. Caveolar constituents, caveolin1, cavin1, and cavin2, co-localized with PgP in these fractions indicating the two pools contained caveolae. A chaperone (Hsc71), protein disulfide isomerase and endosomal/lysosomal sorting proteins (Rab5, Rab11a) also co-fractionated with PgP in the gradients. These data suggest signaling pathways with a potential role in post-translational regulation of PgP activity at the BBB.
This model depicts two types of P-glycoprotein (PgP)-containing caveolae. Rat brain microvessels contain two unique pools of PgP that are of different densities. Each pool co-fractionates with three caveolar proteins suggesting there are two populations of caveolae that contain PgP. The two caveolar populations differ in density indicating they have a different structure, lipoprotein content or intracellular location. Our model includes two newly identified PgP-binding partners, Hsc71 and protein disulfide isomerase, in rat brain microvessels.

3.2389 Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells
De Vrij, J. et al
*Int. J. Cancer, **137**, 1630-1642 (2015)*

Glioblastoma multiforme (GBM) is the most common primary brain tumor and is without exception lethal. GBMs modify the immune system, which contributes to the aggressive nature of the disease. Particularly, cells of the monocytic lineage, including monocytes, macrophages and microglia, are affected. We investigated the influence of GBM-derived extracellular vesicles (EVs) on the phenotype of monocytic cells. Proteomic profiling showed GBM EVs to be enriched with proteins functioning in extracellular matrix interaction and leukocyte migration. GBM EVs appeared to skew the differentiation of peripheral blood-derived monocytes to alternatively activated/M2-type macrophages. This was observed for EVs from an established cell line, as well as for EVs from primary cultures of GBM stem-like cells (GSCs). Unlike EVs of non-GBM origin, GBM EVs induced modified expression of cell surface proteins, modified cytokine secretion (*e.g.*, an increase in vascular endothelial growth factor and IL-6) and increased phagocytic capacity of the macrophages. Most pronounced effects were observed upon incubation with EVs from mesenchymal GSCs. GSC EVs also affected primary human microglia, resulting in increased expression of Membrane type 1-matrix metalloproteinase, a marker for GBM microglia and functioning as tumor-supportive factor. In conclusion, GBM-derived EVs can modify cells of the monocytic lineage, which acquire characteristics that resemble the tumor-supportive phenotypes observed in patients.

3.2390 AP-1/σ1B-Dependent SV Protein Recycling Is Regulated in Early Endosomes and Is Coupled to AP-2 Endocytosis
Kratzke, M., Candiello, E., Schmidt, B., Jahn, O. and Schu, P.
*Mol. Neurobiol., **52**, 142-161 (2015)*

Adaptor protein (AP)-1/σ1B$^{-/-}$ mice have reduced synaptic-vesicle (SV) recycling and increased endosomes. Mutant mice have impaired spatial memory, and σ1B-deficient humans have a severe mental retardation. In order to define these σ1B$^{-/-}$ 'bulk' endosomes and to determine their functions in SV recycling, we developed a protocol to separate them from the majority of the neuronal endosomes. The σ1B$^{-/-}$ 'bulk' endosomes proved to be classic early endosomes with an increase in the phospholipid phosphatidylinositol 3-phosphate (PI-3-P), which recruits proteins mediating protein sorting out of early endosomes into different routes. σ1B deficiency induced alterations in the endosomal proteome reveals two major functions: SV protein storage and sorting into endolysosomes. Alternative endosomal recycling pathways are not up-regulated, but certain SV proteins are misrouted. Tetraspanins are enriched in σ1B$^{-/-}$ synaptosomes, but not in their endosomes or in their clathrin-coated-vesicles (CCVs), indicating AP-1/σ1B-dependent sorting. Synapses contain also more AP-2 CCV, although it is expected that they contain less due to reduced SV recycling. Coat composition of these AP-2 CCVs is altered, and thus, they represent a subpopulation of AP-2 CCVs. Association of calmodulin-dependent protein kinase (CaMK)-IIα, −δ and casein kinase (CK)-IIα with the endosome/SV pool is altered, as well as 14-3-3η, indicating

changes in specific signalling pathways regulating synaptic plasticity. The accumulation of early endosomes and endocytotic AP-2 CCV indicates the regulation of SV recycling via early endosomes by the interdependent regulation of AP-2-mediated endocytosis and AP-1/σ1B-mediated SV reformation.

3.2391 Presenilin 1 Maintains Lysosomal Ca2+ Homeostasis via TRPML1 by Regulating vATPase-Mediated Lysosome Acidification

Lee, J-H., McBrayer, M.K., Wolfe, D.K., Haslett, L.J., Kumar, A., Sato, Y., Lie, P.P.Y., Mohan, P., Coffey, E.E., Kompella, U., Mitchell, C.H., Lloyd-Evans, E. and Nixon, R.A.
*Cell Reports, **12**, 1430-1444 (2015)*

Presenilin 1 (PS1) deletion or Alzheimer's disease (AD)-linked mutations disrupt lysosomal acidification and proteolysis, which inhibits autophagy. Here, we establish that this phenotype stems from impaired glycosylation and instability of vATPase V0a1 subunit, causing deficient lysosomal vATPase assembly and function. We further demonstrate that elevated lysosomal pH in Presenilin 1 knockout (PS1KO) cells induces abnormal Ca^{2+} efflux from lysosomes mediated by TRPML1 and elevates cytosolic Ca^{2+}. In WT cells, blocking vATPase activity or knockdown of either PS1 or the V0a1 subunit of vATPase reproduces all of these abnormalities. Normalizing lysosomal pH in PS1KO cells using acidic nanoparticles restores normal lysosomal proteolysis, autophagy, and Ca^{2+} homeostasis, but correcting lysosomal Ca^{2+} deficits alone neither re-acidifies lysosomes nor reverses proteolytic and autophagic deficits. Our results indicate that vATPase deficiency in PS1 loss-of-function states causes lysosomal/autophagy deficits and contributes to abnormal cellular Ca^{2+} homeostasis, thus linking two AD-related pathogenic processes through a common molecular mechanism.

3.2392 PAQR3 modulates cholesterol homeostasis by anchoring Scap/SREBP complex to the Golgi apparatus

Xu, D., Wang, Z., Zhang, Y., Jiang, W., Pan, Y., Song, B-L. and Chen, Y.
*Nature Communications, **6**:8100 (2015)*

Cholesterol biosynthesis is regulated by transcription factors SREBPs and their escort protein Scap. On sterol depletion, Scap/SREBP complex is transported from endoplasmic reticulum (ER) to the Golgi apparatus where SREBP is activated. Under cholesterol sufficient condition, Insigs act as anchor proteins to retain Scap/SREBP in the ER. However, the anchor protein of Scap/SREBP in the Golgi is unknown. Here we report that a Golgi-localized membrane protein progestin and adipoQ receptors 3 (PAQR3) interacts with Scap and SREBP and tethers them to the Golgi. PAQR3 promotes Scap/SREBP complex formation, potentiates SREBP processing and enhances lipid synthesis. The mutually exclusive interaction between Scap and PAQR3 or Insig-1 is regulated by cholesterol level. PAQR3 knockdown in liver blunts SREBP pathway and decreases hepatic cholesterol content. Disrupting the interaction of PAQR3 with Scap/SREBP by a synthetic peptide inhibits SREBP processing and activation. Thus, PAQR3 regulates cholesterol homeostasis by anchoring Scap/SREBP to the Golgi and disruption of such function reduces cholesterol biosynthesis.

3.2393 A Breast Cell Atlas: Organelle analysis of the MDA-MB-231 cell line by density-gradient fractionation using isotopic marking and label-free analysis

Sandin, M., Antberg, L., Levander, F. and James, P.
*EuPA Open Proteomics, **8**, 68-77 (2015)*

Protein translocation between organelles in the cell is an important process that regulates many cellular functions. However, organelles can rarely be isolated to purity so several methods have been developed to analyse the fractions obtained by density gradient centrifugation. We present an analysis of the distribution of proteins amongst organelles in the human breast cell line, MDA-MB-231 using two approaches: an isotopic labelling and a label-free approach.

3.2394 Strain-Dependent Effect of Macroautophagy on Abnormally Folded Prion Protein Degradation in Infected Neuronal Cells

Ishibashi, D., Homma, T., Nakagaki, T., Fuse, t., sano, K., Takatsuki, H., Atarashi, R. and Nishida, N.
*PloS One, **10(9)**, e0137958 (2015)*

Prion diseases are neurodegenerative disorders caused by the accumulation of abnormal prion protein (PrP^{Sc}) in the central nervous system. With the aim of elucidating the mechanism underlying the accumulation and degradation of PrP^{Sc}, we investigated the role of autophagy in its degradation, using

cultured cells stably infected with distinct prion strains. The effects of pharmacological compounds that inhibit or stimulate the cellular signal transduction pathways that mediate autophagy during PrP^{Sc} degradation were evaluated. The accumulation of PrP^{Sc} in cells persistently infected with the prion strain Fukuoka-1 (FK), derived from a patient with Gerstmann–Sträussler–Scheinker syndrome, was significantly increased in cultures treated with the macroautophagy inhibitor 3-methyladenine (3MA) but substantially reduced in those treated with the macroautophagy inducer rapamycin. The decrease in FK-derived PrP^{Sc} levels was mediated, at least in part, by the phosphatidylinositol 3-kinase/MEK signalling pathway. By contrast, neither rapamycin nor 3MA had any apparently effect on PrP^{Sc} from either the 22L or the Chandler strain, indicating that the degradation of PrP^{Sc} in host cells might be strain-dependent.

3.2395 An efficient two-step subcellular fractionation method for the enrichment of insulin granules from INS-1 cells

Hen, Y., Xia, Z., Wang, L., Yu, Y., Liu, P., Song, E. and Xu, T.
Biophys. Rep., ***1(1)***, *34-40 (2015)*

Insulin is one of the key regulators for blood glucose homeostasis. More than 99% of insulin is secreted from the pancreatic β-cells. Within each β-cell, insulin is packaged and processed in insulin secretary granules (ISGs) before its exocytosis. Insulin secretion is a complicated but well-organized dynamic process that includes the budding of immature ISGs (iISGs) from the *trans*-Golgi network, iISG maturation, and mature ISG (mISG) fusion with plasma membrane. However, the molecular mechanisms involved in this process are largely unknown. It is therefore crucial to separate and enrich iISGs and mISGs before determining their distinct characteristics and protein contents. Here, we developed an efficient two-step subcellular fractionation method for the enrichment of iISGs and mISGs from INS-1 cells: OptiPrep gradient purification followed by Percoll solution purification. We demonstrated that by using this method, iISGs and mISGs can be successfully distinguished and enriched. This method can be easily adapted to investigate SGs in other cells or tissues, thereby providing a useful tool for elucidating the mechanisms of granule maturation and secretion.

3.2396 Identification of Host Cell Factors Associated with Astrovirus Replication in Caco-2 Cells

Murillo, A., Vera-Estrella, R., Barkla, B.J., Mendez, E. and Arias, C.F.
J. Virol., ***89(20)***, *10359-10370 (2015)*

Astroviruses are small, nonenveloped viruses with a single-stranded positive-sense RNA genome causing acute gastroenteritis in children and immunocompromised patients. Since positive-sense RNA viruses have frequently been found to replicate in association with membranous structures, in this work we characterized the replication of the human astrovirus serotype 8 strain Yuc8 in Caco-2 cells, using density gradient centrifugation and free-flow zonal electrophoresis (FFZE) to fractionate cellular membranes. Structural and nonstructural viral proteins, positive- and negative-sense viral RNA, and infectious virus particles were found to be associated with a distinct population of membranes separated by FFZE. The cellular proteins associated with this membrane population in infected and mock-infected cells were identified by tandem mass spectrometry. The results indicated that membranes derived from multiple cell organelles were present in the population. Gene ontology and protein-protein interaction network analysis showed that groups of proteins with roles in fatty acid synthesis and ATP biosynthesis were highly enriched in the fractions of this population in infected cells. Based on this information, we investigated by RNA interference the role that some of the identified proteins might have in the replication cycle of the virus. Silencing of the expression of genes involved in cholesterol (*DHCR7*, *CYP51A1*) and fatty acid (*FASN*) synthesis, phosphatidylinositol (*PI4KIIIβ*) and inositol phosphate (*ITPR3*) metabolism, and RNA helicase activity (*DDX23*) significantly decreased the amounts of Yuc8 genomic and antigenomic RNA, synthesis of the structural protein VP90, and virus yield. These results strongly suggest that astrovirus RNA replication and particle assembly take place in association with modified membranes potentially derived from multiple cell organelles.

3.2397 UBXN2A regulates nicotinic receptor degradation by modulating the E3 ligase activity of CHIP

Teng, Y., Rezvani, K. and De Biasi, M.
Biochem. Pharmacol., ***97***, *518-530 (2015)*

Neuronal nicotinic acetylcholine receptors (nAChRs) containing the α3 subunit are known for their prominent role in normal ganglionic transmission while their involvement in the mechanisms underlying nicotine addiction and smoking-related disease has been emerging only in recent years. The amount of information available on the maturation and trafficking of α3-containing nAChRs is limited. We

previously showed that UBXN2A is a p97 adaptor protein that facilitates the maturation and trafficking of α3-containing nAChRs. Further investigation of the mechanisms of UBXN2A actions revealed that the protein interacts with CHIP (carboxyl terminus of Hsc70 interacting protein), whose ubiquitin E3 ligase activity regulates the degradation of several disease-related proteins. We show that CHIP displays E3 ligase activity toward the α3 nAChR subunit and contributes to its ubiquitination and subsequent degradation. UBXN2A interferes with CHIP-mediated ubiquitination of α3 and protects the nicotinic receptor subunit from endoplasmic reticulum associated degradation (ERAD). UBXN2A also cross-talks with VCP/p97 and HSC70/HSP70 proteins in a complex where α3 is likely to be targeted by CHIP. Overall,we identify CHIP as an E3 ligase for α3 and UBXN2A as a protein that may efficiently regulate the stability of CHIP's client substrates.

3.2398 Tumour-derived exosomes: Tiny envelopes for big stories
Miller, I.V. and Grunewald, T.G.P.
*Biol. Cell, **107**, 287-305 (2015)*

The discovery of exosomes, which are small, 30–100 nm sized extracellular vesicles that are released by virtual all cells, has initiated a rapidly expanding and vibrant research field. Current investigations are mainly directed toward the role of exosomes in intercellular communication and their potential value as biomarkers for a broad set of diseases. By horizontal transfer of molecular information such as micro RNAs, messenger RNAs or proteins, as well as by receptor–cell interactions, exosomes are capable to mediate the reprogramming of surrounding cells. Herein, we review how especially cancer cells take advantage of this mechanism to influence their microenvironment in favour of immune escape, therapy resistance, tumour growth and metastasis. Moreover, we provide a comprehensive microarray analysis ($n > 1970$) to study the expression patterns of genes known to be intimately involved in exosome biogenesis across 26 different cancer entities and a normal tissue atlas. Consistent with the elevated production of exosomes observed in cancer patient plasma, we found a significant overexpression especially of RAB27A, CHMP4C and SYTL4 in the corresponding cancer entities as compared to matched normal tissues. Finally, we discuss the immune-modulatory and anti-tumorigenic functions of exosomes as well as innovative approaches to specifically target the exosomal circuits in experimental cancer therapy.

3.2399 Ebola Virus Glycoprotein Promotes Enhanced Viral Egress by Preventing Ebola VP40 From Associating With the Host Restriction Factor BST2/Tetherin
Gustin, J.K., Bai, Y., Moses, A.V. and Douglas, J.L.
*Journal of Infectious Disease, **212**, Suppl.2, S181-S190 (2015)*

Background. BST2/tetherin is an innate immune molecule with the unique ability to restrict the egress of human immunodeficiency virus (HIV) and other enveloped viruses, including Ebola virus (EBOV). Coincident with this discovery was the finding that the HIV Vpu protein down-regulates BST2 from the cell surface, thereby promoting viral release. Evidence suggests that the EBOV envelope glycoprotein (GP) also counteracts BST2, although the mechanism is unclear.
Results. We find that total levels of BST2 remain unchanged in the presence of GP, whereas surface BST2 is significantly reduced. GP is known to sterically mask surface receptors via its mucin domain. Our evaluation of mutant GP molecules indicate that masking of BST2 by GP is probably responsible for the apparent surface BST2 down-regulation; however, this masking does not explain the observed virus-like particle egress enhancement. We discovered that VP40 coimmunoprecipitates and colocalizes with BST2 in the absence but not in the presence of GP.
Conclusions. These results suggest that GP may overcome the BST2 restriction by blocking an interaction between VP40 and BST2. Furthermore, we have observed that GP may enhance BST2 incorporation into virus-like particles. Understanding this novel EBOV immune evasion strategy will provide valuable insights into the pathogenicity of this deadly pathogen.

3.2400 Rapid multiplex analysis of lipid raft components with single-cell resolution
Schatzlmaier, P., Supper, V., Göschl, L., Zwiritz, A., Eckerstorfer, P., Ellmeier, W., Huppa, J.B. and Stockinger, H.
*Sci. Signal, **8(395)**, rs11 (2015)*

Lipid rafts are dynamic regions of membranes that are involved in cell signaling but are challenging to study because of their small size and dynamic nature. Schatzlmaier *et al*. found that lipid raft components became associated with nuclei during lysis of cells as the cells passed through a detergent-containing layer in a gradient. In experiments with lymphocytes, the authors demonstrated that this association enabled the

quantitative analysis by flow cytometry of the composition of lipid rafts and of the dynamic association of proteins with these membrane microdomains at single-cell resolution.

3.2401 PLD1 participates in BDNF-induced signalling in cortical neurons
Ammar, M.R., Thahouly, T., Hanauer, A., Stegner, D., Nieswandt, b. and Vitale, N.
*Scientific Reports, **5**:14778 (2015)*

The brain-derived neurotrophic factor BDNF plays a critical role in neuronal development and the induction of L-LTP at glutamatergic synapses in several brain regions. However, the cellular and molecular mechanisms underlying these BDNF effects have not been firmly established. Using in vitro cultures of cortical neurons from knockout mice for Pld1 and Rsk2, BDNF was observed to induce a rapid RSK2-dependent activation of PLD and to stimulate BDNF ERK1/2-CREB and mTor-S6K signalling pathways, but these effects were greatly reduced in Pld1−/− neurons. Furthermore, phospho-CREB did not accumulate in the nucleus, whereas overexpression of PLD1 amplified the BDNF-dependent nuclear recruitment of phospho-ERK1/2 and phospho-CREB. This BDNF retrograde signalling was prevented in cells silenced for the scaffolding protein PEA15, a protein which complexes with PLD1, ERK1/2, and RSK2 after BDNF treatment. Finally PLD1, ERK1/2, and RSK2 partially colocalized on endosomal structures, suggesting that these proteins are part of the molecular module responsible for BDNF signalling in cortical neurons.

3.2402 Dynamic tubulation of mitochondria drives mitochondrial network formation
Wang, C., Du, W., Su, Q., Zhu, M., Feng, P., Li, Y., Zhou, Y., Mi, N., Zhu, Y., Jiang, D., Zhang, S., Zhang, Z., Sun, Y. and Yu, L.
*Cell Research, **25**, 1108-1120 (2015)*

Mitochondria form networks. Formation of mitochondrial networks is important for maintaining mitochondrial DNA integrity and interchanging mitochondrial material, whereas disruption of the mitochondrial network affects mitochondrial functions. According to the current view, mitochondrial networks are formed by fusion of individual mitochondria. Here, we report a new mechanism for formation of mitochondrial networks through KIF5B-mediated dynamic tubulation of mitochondria. We found that KIF5B pulls thin, highly dynamic tubules out of mitochondria. Fusion of these dynamic tubules, which is mediated by mitofusins, gives rise to the mitochondrial network. We further demonstrated that dynamic tubulation and fusion is sufficient for mitochondrial network formation, by reconstituting mitochondrial networks *in vitro* using purified fusion-competent mitochondria, recombinant KIF5B, and polymerized microtubules. Interestingly, KIF5B only controls network formation in the peripheral zone of the cell, indicating that the mitochondrial network is divided into subzones, which may be constructed by different mechanisms. Our data not only uncover an essential mechanism for mitochondrial network formation, but also reveal that different parts of the mitochondrial network are formed by different mechanisms.

3.2403 Live-Cell Imaging of Phagosome Motility in Primary Mouse RPE Cells
Hazim, R., Jiang, M., Esteve-Rudd, J., Diemer, T., Lopes, V.S. and Williams, D.S.
*Exp. Med. Biol., **854**, 751-755 (2015)*

The retinal pigment epithelium (RPE) is a post-mitotic epithelial monolayer situated between the light-sensitive photoreceptors and the choriocapillaris. Given its vital functions for healthy vision, the RPE is a primary target for insults that result in blinding diseases, including age-related macular degeneration (AMD). One such function is the phagocytosis and digestion of shed photoreceptor outer segments. In the present study, we examined the process of trafficking of outer segment disk membranes in live cultures of primary mouse RPE, using high speed spinning disk confocal microscopy. This approach has enabled us to track phagosomes, and determine parameters of their motility, which are important for their efficient degradation.

3.2404 Apolipoprotein E Regulates Amyloid Formation within Endosomes of Pigment Cells
Van Niel, G., bergam, P., Di Cicco, A., Hurbain, I., Lo Cicero, A., Dingli, F., Palmulli, R., Fort, C., Potier, M.C., Schurgers, L.J., Loew, D., Levy, D. and Raposo, G.
*Cell reports, **13(1)**, 43-51 (2015)*

Accumulation of toxic amyloid oligomers is a key feature in the pathogenesis of amyloid-related diseases. Formation of mature amyloid fibrils is one defense mechanism to neutralize toxic prefibrillar oligomers.

This mechanism is notably influenced by *apolipoprotein E* variants. Cells that produce mature amyloid fibrils to serve physiological functions must exploit specific mechanisms to avoid potential accumulation of toxic species. Pigment cells have tuned their endosomes to maximize the formation of functional amyloid from the protein PMEL. Here, we show that ApoE is associated with intraluminal vesicles (ILV) within endosomes and remain associated with ILVs when they are secreted as exosomes. ApoE functions in the ESCRT-independent sorting mechanism of PMEL onto ILVs and regulates the endosomal formation of PMEL amyloid fibrils in vitro and in vivo. This process secures the physiological formation of amyloid fibrils by exploiting ILVs as amyloid nucleating platforms.

3.2405 **Proteomic analysis of extracellular vesicles derived from Mycobacterium tuberculosis**
Lee, J., Kim, S-H., Choi, D-S., Lee, J.S., Kim, D-K., Go, G., Park, S-M., Kim, S.H., Shin, J.H., Chang, C.L. and Gho, Y.S.
Proteomics, ***15(19)***, *3331-3337 (2015)*

The release of extracellular vesicles, also known as outer membrane vesicles, membrane vesicles, exosomes, and microvesicles, is an evolutionarily conserved phenomenon from bacteria to eukaryotes. It has been reported that *Mycobacterium tuberculosis* releases extracellular vesicles harboring immunologically active molecules, and these extracellular vesicles have been suggested to be applicable in vaccine development and biomarker discovery. However, the comprehensive proteomic analysis has not been performed for *M. tuberculosis* extracellular vesicles. In this study, we identified a total of 287 vesicular proteins by four LC-MS/MS analyses with high confidence. In addition, we identified several vesicular proteins associated with the virulence of *M. tuberculosis*. This comprehensive proteome profile will help elucidate the pathogenic mechanism of *M. tuberculosis*. The data have been deposited to the ProteomeXchange with identifier PXD001160 (http://proteomecentral.proteomexchange.org/dataset/PXD001160).

3.2406 **G206D Mutation of Presenilin-1 Reduces Pen2 Interaction, Increases Aβ42/Aβ40 Ratio and Elevates ER Ca2+ Accumulation**
Chen, W-T., Hsieh, Y-F., Huang, Y-J., Lin, C-C., Lin, Y-T., Liu, Y-C., Lien, C-C and Cheng, I-H-J.
Mol. Neurobiol.,***52***, *1835-1849 (2015)*

Early-onset familial Alzheimer's disease (AD) is most commonly associated with the mutations in presenilin-1 (PS1). PS1 is the catalytic component of the γ-secretase complex, which cleaves amyloid precursor protein to produce amyloid-β (Aβ), the major cause of AD. Presenilin enhancer 2 (Pen2) is critical for activating γ-secretase and exporting PS1 from endoplasmic reticulum (ER). Among all the familial AD-linked PS1 mutations, mutations at the G206 amino acid are the most adjacent position to the Pen2 binding site. Here, we characterized the effect of a familial AD-linked PS1 G206D mutation on the PS1-Pen2 interaction and the accompanied alteration in γ-secretase-dependent and -independent functions. We found that the G206D mutation reduced PS1-Pen2 interaction, but did not abolish γ-secretase formation and PS1 endoproteolysis. For γ-secretase-dependent function, the G206D mutation increased $A\beta_{42}$ production but not Notch cleavage. For γ-secretase-independent function, this mutation disrupted the ER calcium homeostasis but not lysosomal calcium homeostasis and autophagosome maturation. Impaired ER calcium homeostasis may due to the reduced mutant PS1 level in the ER. Although this mutation did not alter the cell survival under stress, both increased $A\beta_{42}$ ratio and disturbed ER calcium regulation could be the mechanisms underlying the pathogenesis of the familial AD-linked PS1 G206D mutation.

3.2407 **Proteomics in neurodegenerative diseases: Methods for obtaining a closer look at the neuronal proteome**
Plum, S., Steinbach, S., Abel, L., Marcus, K., helling, S. and may, C.
Proteomics Clin. Appl., ***9(9-10)***, *848-871 (2015)*

The analysis of brain function in normal aging and neurodegenerative, psychiatric, and neurological diseases has long been a subject of interest and has historically been investigated through descriptive analysis of macroscopic or microscopic observations. It is now possible to characterize brain cells, such as neurons and glial cells, or even their subcellular components, at the molecular level. This ability enables researchers to more closely examine brain cell specific molecular pathways to elucidate distinct brain functions. Furthermore, the analysis of neuronal maintenance and disease-causing effects is a central component of neurological investigations, which include proteomic approaches. Proteomics allows the identification of thousands of proteins through descriptive and comparative analyses and can provide a detailed overview of a distinct cellular state. Such analyses often require the isolation of individual cell

types or subcellular components to investigate specific questions. This review provides an overview of the currently applied state-of-the-art prefractionation strategies in this field.

3.2408 Proteoglycans support proper granule formation in pancreatic acinar cells
Aroso, M., Agricola, B., Hacker, C. and Schrader, M
Histochem. Cell Biol., ***144****, 331-346 (2015)*

Zymogen granules (ZG) are specialized organelles in the exocrine pancreas which allow digestive enzyme storage and regulated secretion. The molecular mechanisms of their biogenesis and the sorting of zymogens are still incompletely understood. Here, we investigated the role of proteoglycans in granule formation and secretion of zymogens in pancreatic AR42J cells, an acinar model system. Cupromeronic Blue cytochemistry and biochemical studies revealed an association of proteoglycans primarily with the granule membrane. Removal of proteoglycans by carbonate treatment led to a loss of membrane curvature indicating a supportive role in the maintenance of membrane shape and stability. Chemical inhibition of proteoglycan synthesis impaired the formation of normal electron-dense granules in AR42J cells and resulted in the formation of unusually small granule structures. These structures still contained the zymogen carboxypeptidase, a cargo molecule of secretory granules, but migrated to lighter fractions after density gradient centrifugation. Furthermore, the basal secretion of amylase was increased in AR42J cells after inhibitor treatment. In addition, irregular-shaped granules appeared in pancreatic lobules. We conclude that the assembly of a proteoglycan scaffold at the ZG membrane is supporting efficient packaging of zymogens and the proper formation of stimulus-competent storage granules in acinar cells of the pancreas.

3.2409 mRNA Targeting to Endoplasmic Reticulum Precedes Ago Protein Interaction and MicroRNA (miRNA)-mediated Translation Repression in Mammalian Cells
Barman, B. and Bhattacharyya, S.N.
J. Biol. Chem., ***290(41)****, 24650-24656 (2015)*

MicroRNA (miRNA) binds to the 3′-UTR of its target mRNAs to repress protein synthesis. Extensive research was done to understand the mechanism of miRNA-mediated repression in animal cells. Considering the progress in understanding the mechanism, information about the subcellular sites of miRNA-mediated repression is surprisingly limited. In this study, using an inducible expression system for an miRNA target message, we have delineated how a target mRNA passes through polysome association and Ago2 interaction steps on rough endoplasmic reticulum (ER) before the miRNA-mediated repression sets in. From this study, *de novo* formed target mRNA localization to the ER-bound polysomes manifested as the earliest event, which is followed by Ago2 micro-ribonucleoprotein binding, and translation repression of target message. Compartmentalization of this process to rough ER membrane ensures enrichment of miRNA-targeted messages and micro-ribonucleoprotein components on ER upon reaching a steady state.

3.2410 Role of Pex21p for Piggyback Import of Gpd1p and Pnc1p into Peroxisomes of Saccharomyces cerevisiae
Effelsberg, D., Cruz-Zaragoza, L.D., Tonillo, J., Schliebs, W. and Erdmann, R.
J. Biol. Chem., ***290(42)****, 25333-25342 (2015)*

Proteins designated for peroxisomal protein import harbor one of two common peroxisomal targeting signals (PTS). In the yeast *Saccharomyces cerevisiae*, the oleate-induced PTS2-dependent import of the thiolase Fox3p into peroxisomes is conducted by the soluble import receptor Pex7p in cooperation with the auxiliary Pex18p, one of two supposedly redundant PTS2 co-receptors. Here, we report on a novel function for the co-receptor Pex21p, which cannot be fulfilled by Pex18p. The data establish Pex21p as a general co-receptor in PTS2-dependent protein import, whereas Pex18p is especially important for oleate-induced import of PTS2 proteins. The glycerol-producing PTS2 protein glycerol-3-phosphate dehydrogenase Gpd1p shows a tripartite localization in peroxisomes, in the cytosol, and in the nucleus under osmotic stress conditions. We show the following: (i) Pex21p is required for peroxisomal import of Gpd1p as well as a key enzyme of the NAD^+ salvage pathway, Pnc1p; (ii) Pnc1p, a nicotinamidase without functional PTS2, is co-imported into peroxisomes by piggyback transport via Gpd1p. Moreover, the specific transport of these two enzymes into peroxisomes suggests a novel regulatory role for peroxisomes under various stress conditions.

3.2411 Membrane vesicle-mediated release of bacterial RNA
Sjöström, A.E., Sandblad, L., Uhlin, B.E. and Wai, S.N.
*Scientific Reports, **5**:15329 (2015)*

Many Gram-negative bacterial species release outer membrane vesicles (OMVs) that interact with the host by delivering virulence factors. Here, we report for the first time that RNA is among the wide variety of bacterial components that are associated with OMVs. To characterize the RNA profiles of bacterial OMVs, we performed RNA deep sequencing analysis using OMV samples isolated from a wild type Vibrio cholerae O1 El Tor strain. The results showed that RNAs originating from intergenic regions were the most abundant. Our findings reveal a hitherto unrecognised feature of OMVs mimicking eukaryotic exosomes and highlight a need to evaluate the potential role of RNA-containing bacterial membrane vesicles in bacteria-host interactions.

3.2412 Noninvasive imaging of radiolabeled exosome-mimetic nanovesicle using 99mTc-HMPAO
Hwang, D.W., Choi, H., jang, S.C., Yoo, M.Y., park, J.Y., Choi, N.E., Oh, H.J., Ha, S., Lee, Y-S., Jeong, J.M., Gho, Y.S. and Lee, D.S.
*Scientific Reports, **5**:15636 (2015)*

Exosomes known as nano-sized extracellular vesicles attracted recent interests due to their potential usefulness in drug delivery. Amid remarkable advances in biomedical applications of exosomes, it is crucial to understand in vivo distribution and behavior of exosomes. Here, we developed a simple method for radiolabeling of macrophage-derived exosome-mimetic nanovesicles (ENVs) with 99mTc-HMPAO under physiologic conditions and monitored in vivo distribution of 99mTc-HMPAO-ENVs using SPECT/CT in living mice. ENVs were produced from the mouse RAW264.7 macrophage cell line and labeled with 99mTc-HMPAO for 1 hr incubation, followed by removal of free 99mTc-HMPAO. SPECT/CT images were serially acquired after intravenous injection to BALB/c mouse. When ENVs were labeled with 99mTc-HMPAO, the radiochemical purity of 99mTc-HMPAO-ENVs was higher than 90% and the expression of exosome specific protein (CD63) did not change in 99mTc-HMPAO-ENVs. 99mTc-HMPAO-ENVs showed high serum stability (90%) which was similar to that in phosphate buffered saline until 5 hr. SPECT/CT images of the mice injected with 99mTc-HMPAO-ENVs exhibited higher uptake in liver and no uptake in brain, whereas mice injected with 99mTc-HMPAO showed high brain uptake until 5 hr. Our noninvasive imaging of radiolabeled-ENVs promises better understanding of the in vivo behavior of exosomes for upcoming biomedical application.

3.2413 Rhodopsin Forms Nanodomains in Rod Outer Segment Disc Membranes of the Cold-Blooded Xenopus laevis
Rakshit, T., Senapati, S., Sinha, S., Whited, A.M. and Park, P.S-H.
*PloS One, **10(10)**, e0141114 (2015)*

Rhodopsin forms nanoscale domains (i.e., nanodomains) in rod outer segment disc membranes from mammalian species. It is unclear whether rhodopsin arranges in a similar manner in amphibian species, which are often used as a model system to investigate the function of rhodopsin and the structure of photoreceptor cells. Moreover, since samples are routinely prepared at low temperatures, it is unclear whether lipid phase separation effects in the membrane promote the observed nanodomain organization of rhodopsin from mammalian species. Rod outer segment disc membranes prepared from the cold-blooded frog *Xenopus laevis* were investigated by atomic force microscopy to visualize the organization of rhodopsin in the absence of lipid phase separation effects. Atomic force microscopy revealed that rhodopsin nanodomains form similarly as that observed previously in mammalian membranes. Formation of nanodomains in ROS disc membranes is independent of lipid phase separation and conserved among vertebrates.

3.2414 Gut microbe-derived extracellular vesicles induce insulin resistance, thereby impairing glucose metabolism in skeletal muscle
Choi, Y., Kwon, Y., Kim, D-K., Jeon, J., Jang, S.C., Wang, T., Ban, M., Kim, M-H., Jeon, S.G., Kim, M-S., Choi, C.S., Jee, Y-K., Gho, Y.S., Ryu, S.H. and Kim, Y-K.
*Scientific Reports, **5**:15878 (2015)*

Gut microbes might influence host metabolic homeostasis and contribute to the pathogenesis of type 2 diabetes (T2D), which is characterized by insulin resistance. Bacteria-derived extracellular vesicles (EVs) have been suggested to be important in the pathogenesis of diseases once believed to be non-infectious.

Here, we hypothesize that gut microbe-derived EVs are important in the pathogenesis of T2D. In vivo administration of stool EVs from high fat diet (HFD)-fed mice induced insulin resistance and glucose intolerance compared to regular diet (RD)-fed mice. Metagenomic profiling of stool EVs by 16S ribosomal DNA sequencing revealed an increased amount of EVs derived from Pseudomonas panacis (phylum Proteobacteria) in HFD mice compared to RD mice. Interestingly, P. panacis EVs blocked the insulin signaling pathway in both skeletal muscle and adipose tissue. Moreover, isolated P. panacis EVs induced typical diabetic phenotypes, such as glucose intolerance after glucose administration or systemic insulin injection. Thus, gut microbe-derived EVs might be key players in the development of insulin resistance and impairment of glucose metabolism promoted by HFD.

3.2415 AIF inhibits tumor metastasis by protecting PTEN from oxidation
Shen, S-M., Guo, M., Xiong, Z., Yu, Y., Zhao, X-Y., Zhang, F-F. and Chen, G-Q.
EMBO Rep., ***16(11)***, *1563-1580 (2015)*

Apoptosis-inducing factor (AIF) exerts dual roles on cell death and survival, but its substrates as a putative oxidoreductase and roles in tumorigenesis remain elusive. Here, we report that AIF physically interacts with and inhibits the oxidation of phosphatase and tensin homolog on chromosome ten (PTEN), a tumor suppressor susceptible for oxidation-mediated inactivation. More intriguingly, we also identify PTEN as a mitochondrial protein and the ectopic expression of mitochondrial targeting sequence-carrying PTEN almost completely inhibits Akt phosphorylation in PTEN-deficient cells. AIF knockdown causes oxidation-mediated inactivation of the lipid phosphatase activity of PTEN, with ensuing activation of Akt kinase, phosphorylation of the Akt substrate GSK-3β, and activation of β-catenin signaling in cancer cells. Through its effect on β-catenin signaling, AIF inhibits epithelial–mesenchymal transition (EMT) and metastasis of cancer cells *in vitro* and in orthotopically implanted xenografts. Accordingly, the expression of AIF is correlated with the survival of human patients with cancers of multiple origins. These results identify PTEN as the substrate of AIF oxidoreductase and reveal a novel function for AIF in controlling tumor metastasis.

3.2416 Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes
Taylor, D.D. and Shah, S.
Methods, ***87***, *3-10 (2015)*

Viable tumor cells actively release vesicles into the peripheral circulation and other biologic fluids, which exhibit proteins and RNAs characteristic of that cell. Our group demonstrated the presence of these extracellular vesicles of tumor origin within the peripheral circulation of cancer patients and proposed their utility for diagnosing the presence of tumors and monitoring their response to therapy in the 1970s. However, it has only been in the past 10 years that these vesicles have garnered interest based on the recognition that they serve as essential vehicles for intercellular communication, are key determinants of the immunosuppressive microenvironment observed in cancer and provide stability to tumor-derived components that can serve as diagnostic biomarkers. To date, the clinical utility of extracellular vesicles has been hampered by issues with nomenclature and methods of isolation. The term “exosomes” was introduced in 1981 to denote any nanometer-sized vesicles released outside the cell and to differentiate them from intracellular vesicles. Based on this original definition, we use “exosomes” as synonymous with “extracellular vesicles.” While our original studies used ultracentrifugation to isolate these vesicles, we immediately became aware of the significant impact of the isolation method on the number, type, content and integrity of the vesicles isolated. In this review, we discuss and compare the most commonly utilized methods for purifying exosomes for post-isolation analyses. The exosomes derived from these approaches have been assessed for quantity and quality of specific RNA populations and specific marker proteins. These results suggest that, while each method purifies exosomal material, there are pros and cons of each and there are critical issues linked with centrifugation-based methods, including co-isolation of non-exosomal materials, damage to the vesicle’s membrane structure and non-standardized parameters leading to qualitative and quantitative variability. The down-stream analyses of these resulting varying exosomes can yield misleading results and conclusions.

3.2417 Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct
Xu, R., Greening, D.W., Rai, A., Ji, H. and Simpson, R.J.
Methods, ***87***, *11-25 (2015)*

Secretion and exchange of extracellular vesicles (EVs) by most cell types is emerging as a fundamental biological process. Although much is known about EVs, there is still a lack of definition as to how many naturally occurring EV subtypes there are and how their properties and functionalities might differ. This vexing issue is critical if EVs are to be fully harnessed for therapeutic applications. To address this question we have developed and describe here a sequential centrifugal ultrafiltration (SCUF) method to examine, in an unbiased manner, what EV subtypes are released *in vitro* into cell culture medium using the human colon carcinoma cell line LIM1863 as a model system. Using the culture medium from ~7.2×10^9 LIM1863 cells, SCUF was performed using hydrophilic PVDF membranes with low protein binding properties (Millipore Durapore™ Ultrafree-CL filters with 0.1, 0.22, 0.45 and 0.65 μm pore size). EV particle sizing was measured using both dynamic light scattering and cryo-electron microscopy. Comparative proteome profiling was performed by GeLC–MS/MS and qualitative protein differences between EV subtypes determined by label-free spectral counting. The results showed essentially two EV subtypes; one subtype (fraction Fn1) comprised heterogeneous EVs with particle diameters of 30–1300 nm, the other (fraction Fn5) being homogeneous EVs of 30–100 nm diameter; based on cryo-EM both EV subtypes were round shaped. Western blot analysis showed Fn5 (SCUF-Exos) contained traditional exosome marker proteins ($Alix^+$, $TSG101^+$, $CD81^+$, $CD63^+$), while Fn1 (SCUF-sMVs) lacked these protein markers. These findings were consistent with sMVs isolated by differential centrifugation (10,000*g*, DC-sMVs) and exosomes (100,000*g* EVs depleted of 10,000*g* material). The buoyant density of sMVs determined by OptiPrep™ density gradient centrifugation was 1.18–1.19 g/mL and exosomes 1.10–1.11 g/mL. Comparative protein profiling of SCUF-Exos/-sMVs revealed 354 and 606 unambiguous protein identifications, respectively, with 256 proteins in common. A salient finding was the first report of 350 proteins uniquely identified in sMVs may of which have the potential to enable discrimination of this EV subtype from exosomes (notably, members of the septin family, kinesin-like protein (KIF23), exportin-2/chromosome segregation like-1 protein (CSE1L), and Rac GTPase-activating protein 1 (RACGAP1)). We report for the first time that both SCUF-Exos and SCUF-sMVs isolated from LIM1863 colon cancer cells induce invasion of recipient NIH3T3 cells. Interestingly, the SCUF-sMVs promote invasion to a significantly greater extent (3-fold) than SCUF-Exos. This analytical SCUF method for fractionating EVs is potentially scalable using tangential flow filtration, thereby providing a solid foundation for future in-depth functional studies of EV subtypes using diverse cell types and functional assays.

3.2418 Human Mitochondrial DNA-Protein Complexes Attach to a Cholesterol-Rich Membrane Structure
Gerhold, J.M., Cansiz-Arda, S., Löhmus, M., Engberg, O., Reyes, A., van Rennes, H., Sanz, A., Holt, I.J., Cooper, H.M. and Spelbrink, J.N.
Scientific Reports, 5:15292 (2015)

The helicase Twinkle is indispensable for mtDNA replication in nucleoids. Previously, we showed that Twinkle is tightly membrane-associated even in the absence of mtDNA, which suggests that Twinkle is part of a membrane-attached replication platform. Here we show that this platform is a cholesterol-rich membrane structure. We fractionated mitochondrial membrane preparations on flotation gradients and show that membrane-associated nucleoids accumulate at the top of the gradient. This fraction was shown to be highly enriched in cholesterol, a lipid that is otherwise low abundant in mitochondria. In contrast, more common mitochondrial lipids, and abundant inner-membrane associated proteins concentrated in the bottom-half of these gradients. Gene silencing of ATAD3, a protein with proposed functions related to nucleoid and mitochondrial cholesterol homeostasis, modified the distribution of cholesterol and nucleoids in the gradient in an identical fashion. Both cholesterol and ATAD3 were previously shown to be enriched in ER-mitochondrial junctions, and we detect nucleoid components in biochemical isolates of these structures. Our data suggest an uncommon membrane composition that accommodates platforms for replicating mtDNA, and reconcile apparently disparate functions of ATAD3. We suggest that mtDNA replication platforms are organized in connection with ER-mitochondrial junctions, facilitated by a specialized membrane architecture involving mitochondrial cholesterol.

3.2419 Signal dependent ER export of lemur tyrosine kinase 2
Butler, E.C. and Bradbury, N.A.
*BMC Cell Biology, **16**:26 (2015)*

Background
The membrane anchored kinase, LMTK2, is a serine/threonine kinase predominantly localized to endosomal compartments. LMTK2 has been shown to be involved in the trafficking of the CFTR ion channel, the androgen receptor, as well as modulating neurodegeneration. As a membrane anchored

protein, LMTK2 must be exported from the ER, yet the mechanisms whereby LMTK2 is sequestered within the ER for efficient export are unknown.

Methods

Sequence analysis of the carboxyl tail of LMTK2 revealed a putative di-acidic ER export motif. Site-directed mutagenesis was utilized to ablate this potential motif. Subcellular fractionation, immunofluorescence microscopy, and transferrin recycling assays were used to determine the consequence of mutating LMTK2's export motif.

Results

Mutation of the di-acidic export motif led to ER retention of LMTK2, and an increase in protein half-life and a concomitant loss of LMTK2 from its appropriate terminal destination. Loss of LMTK2 from endosomal compartments by preventing its release from the ER is linked to a reduction in transferrin recycling.

Conclusions

We have identified a di-acidic ER export motif within the carboxyl tail of the membrane anchored kinase LMTK2. This sequence is used by LMTK2 for its efficient export from the ER.

3.2420 Phospholipid methylation controls Atg32-mediated mitophagy and Atg8 recycling

Sakakibara, K. et al

EMBO J., ***34(21)****, 2703-2719 (2015)*

Degradation of mitochondria via selective autophagy, termed mitophagy, contributes to mitochondrial quality and quantity control whose defects have been implicated in oxidative phosphorylation deficiency, aberrant cell differentiation, and neurodegeneration. How mitophagy is regulated in response to cellular physiology remains obscure. Here, we show that mitophagy in yeast is linked to the phospholipid biosynthesis pathway for conversion of phosphatidylethanolamine to phosphatidylcholine by the two methyltransferases Cho2 and Opi3. Under mitophagy-inducing conditions, cells lacking Opi3 exhibit retardation of Cho2 repression that causes an anomalous increase in glutathione levels, leading to suppression of Atg32, a mitochondria-anchored protein essential for mitophagy. In addition, loss of Opi3 results in accumulation of phosphatidylmonomethylethanolamine (PMME) and, surprisingly, generation of Atg8–PMME, a mitophagy-incompetent lipid conjugate of the autophagy-related ubiquitin-like modifier. Amelioration of Atg32 expression and attenuation of Atg8–PMME conjugation markedly rescue mitophagy in *opi3*-null cells. We propose that proper regulation of phospholipid methylation is crucial for Atg32-mediated mitophagy.

3.2421 The lipid raft proteome of Borrelia burgdorferi

Toledo, A., Perez, A., Coleman, J.L. and benach, J.L.

Proteomics, ***15(21)****, 3662-3675 (2015)*

Eukaryotic lipid rafts are membrane microdomains that have significant amounts of cholesterol and a selective set of proteins that have been associated with multiple biological functions. The Lyme disease agent, *Borrelia burgdorferi*, is one of an increasing number of bacterial pathogens that incorporates cholesterol onto its membrane, and form cholesterol glycolipid domains that possess all the hallmarks of eukaryotic lipid rafts. In this study, we isolated lipid rafts from cultured *B. burgdorferi* as a detergent resistant membrane (DRM) fraction on density gradients, and characterized those molecules that partitioned exclusively or are highly enriched in these domains. Cholesterol glycolipids, the previously known raft-associated lipoproteins OspA and OpsB, and cholera toxin partitioned into the lipid rafts fraction indicating compatibility with components of the DRM. The proteome of lipid rafts was analyzed by a combination of LC-MS/MS or MudPIT. Identified proteins were analyzed *in silico* for parameters that included localization, isoelectric point, molecular mass and biological function. The proteome provided a consistent pattern of lipoproteins, proteases and their substrates, sensing molecules and prokaryotic homologs of eukaryotic lipid rafts. This study provides the first analysis of a prokaryotic lipid raft and has relevance for the biology of *Borrelia*, other pathogenic bacteria, as well as for the evolution of these structures. All MS data have been deposited in the ProteomeXchange with identifier PXD002365

3.2422 Exogenous DNA Loading into Extracellular Vesicles via Electroporation is Size-Dependent and Enables Limited Gene Delivery

Lamichhane, T.N., Raiker, R.S. and Jay, S.M.

Mol Pharmaceut., ***12(10)****, 3650-3657 (2015)*

Extracellular vesicles (EVs) hold immense promise for utilization as biotherapeutics and drug delivery vehicles due to their nature as biological nanoparticles that facilitate intercellular molecular transport. Specifically, EVs have been identified as natural carriers of nucleic acids, sparking interest in their use for gene therapy and RNA interference applications. So far, small RNAs (siRNA and miRNA) have been successfully loaded into EVs for a variety of delivery applications, but the potential use of EVs for DNA delivery has scarcely been explored. Here, we report that exogenous linear DNA can be associated with EVs via electroporation in quantities sufficient to yield an average of hundreds of DNA molecules per vesicle. We determined that loading efficiency and capacity of DNA in EVs is dependent on DNA size, with linear DNA molecules less than 1000 bp in length being more efficiently associated with EVs compared to larger linear DNAs and plasmid DNAs using this approach. We further showed that EV size is also determinant with regard to DNA loading, as larger microvesicles encapsulated more linear and plasmid DNA than smaller, exosome-like EVs. Additionally, we confirmed the ability of EVs to transfer foreign DNA loaded via electroporation into recipient cells, although functional gene delivery was not observed. These results establish critical parameters that inform the potential use of EVs for gene therapy and, in agreement with other recent results, suggest that substantial barriers must be overcome to establish EVs as broadly applicable DNA delivery vehicles.

3.2423 Proteome Profiles of Outer Membrane Vesicles and Extracellular Matrix of Pseudomonas aeruginosa Biofilms

Couto, N., Schooling, S.R., Dutcher, J.R. and Barber, J.
J. Proteome Res., ***14(10)***, *4207-4222 (2015)*

In the present work, two different proteomic platforms, gel-based and gel-free, were used to map the matrix and outer membrane vesicle exoproteomes of *Pseudomonas aeruginosa* PAO1 biofilms. These two proteomic strategies allowed us a confident identification of 207 and 327 proteins from enriched outer membrane vesicles and whole matrix isolated from biofilms. Because of the physicochemical characteristics of these subproteomes, the two strategies showed complementarity, and thus, the most comprehensive analysis of *P. aeruginosa* exoproteome to date was achieved. Under our conditions, outer membrane vesicles contribute approximately 20% of the whole matrix proteome, demonstrating that membrane vesicles are an important component of the matrix. The proteomic profiles were analyzed in terms of their biological context, namely, a biofilm. Accordingly relevant metabolic processes involved in cellular adaptation to the biofilm lifestyle as well as those related to *P. aeruginosa* virulence capabilities were a key feature of the analyses. The diversity of the matrix proteome corroborates the idea of high heterogeneity within the biofilm; cells can display different levels of metabolism and can adapt to local microenvironments making this proteomic analysis challenging. In addition to analyzing our own primary data, we extend the analysis to published data by other groups in order to deepen our understanding of the complexity inherent within biofilm populations.

3.2424 TRPC6 specifically interacts with APP to inhibit its cleavage by γ-secretase and reduce Aβ production

Wang, J., Lu, R., yang, J., Li, H., He, Z., Jing, N., Wang, X. and Wang, Y.
Nature Communications, ***6****:8876 (2015)*

Generation of β-amyloid (Aβ) peptide in Alzheimer's disease involves cleavage of amyloid precursor protein (APP) by γ-secretase, a protease known to cleave several substrates, including Notch. Finding specific modulators for γ-secretase could be a potential avenue to treat the disease. Here, we report that transient receptor potential canonical (TRPC) 6 specifically interacts with APP leading to inhibition of its cleavage by γ-secretase and reduction in Aβ production. TRPC6 interacts with APP (C99), but not with Notch, and prevents C99 interaction with presenilin 1 (PS1). A fusion peptide derived from TRPC6 also reduces Aβ levels without effect on Notch cleavage. Crossing *APP/PS1* mice with *TRPC6* transgenic mice leads to a marked reduction in both plaque load and Aβ levels, and improvement in structural and behavioural impairment. Thus, TRPC6 specifically modulates γ-secretase cleavage of APP and preventing APP (C99) interaction with PS1 via TRPC6 could be a novel strategy to reduce Aβ formation.

3.2425 Integrin α5 Suppresses the Phosphorylation of Epidermal Growth Factor Receptor and Its Cellular Signaling of Cell Proliferation via N-Glycosylation

Hang, Q., Isaji, T., Hou, S., Im, S., Fukuda, T. and Gu, J.
J. Biol. Chem., ***290(49)***, *29345-29360 (2015)*

Integrin α5β1-mediated cell adhesion regulates a multitude of cellular responses, including cell proliferation, survival, and cross-talk between different cellular signaling pathways. Integrin α5β1 is known to convey permissive signals enabling anchorage-dependent receptor tyrosine kinase signaling. However, the effects of integrin α5β1 on cell proliferation are controversial, and the molecular mechanisms involved in the regulation between integrin α5β1 and receptor tyrosine kinase remain largely unclear. Here we show that integrin α5 functions as a negative regulator of epidermal growth factor receptor (EGFR) signaling through its *N*-glycosylation. Expression of WT integrin α5 suppresses the EGFR phosphorylation and internalization upon EGF stimulation. However, expression of the *N*-glycosylation mutant integrin α5, S3–5, which contains fewer *N*-glycans, reversed the suppression of the EGFR-mediated signaling and cell proliferation. In a mechanistic manner, WT but not S3–5 integrin α5 forms a complex with EGFR and glycolipids in the low density lipid rafts, and the complex formation is disrupted upon EGF stimulation, suggesting that the *N*-glycosylation of integrin α5 suppresses the EGFR activation through promotion of the integrin α5-glycolipids-EGFR complex formation. Furthermore, consistent restoration of those *N*-glycans on the Calf-1,2 domain of integrin α5 reinstated the inhibitory effects as well as the complex formation with EGFR. Taken together, these data are the first to demonstrate that EGFR activation can be regulated by the *N*-glycosylation of integrin α5, which is a novel molecular paradigm for the cross-talk between integrins and growth factor receptors.

3.2426 Luman is involved in osteoclastogenesis through the regulation of DC-STAMP expression, stability and localization

Kanemoto, S., Kobayashi, Y., yamashita, T., Miyamoto, T., cui, M., asada, R., Cui, X., Hino, K., kaneko, M., Takai, T., matsuhisa, K., tahahashi, N and Imaizumi, K.
J. Cell Sci., ***128****, 4353-4365 (2015)*

Luman (also known as CREB3) is a type-II transmembrane transcription factor belonging to the OASIS family that localizes to the endoplasmic reticulum (ER) membrane under normal conditions. In response to ER stress, OASIS-family members are subjected to regulated intramembrane proteolysis (RIP), following which the cleaved N-terminal fragments translocate to the nucleus. In this study, we show that treatment of bone marrow macrophages (BMMs) with cytokines – macrophage colony-stimulating factor (M-CSF) and RANKL (also known as TNFSF11) – causes a time-dependent increase in Luman expression, and that Luman undergoes RIP and becomes activated during osteoclast differentiation. Small hairpin (sh)RNA-mediated knockdown of Luman in BMMs prevented the formation of multinucleated osteoclasts, concomitant with the suppression of DC-STAMP, a protein that is essential for cell–cell fusion in osteoclastogenesis. The N-terminus of Luman facilitates promoter activity of DC-STAMP, resulting in upregulation of DC-STAMP expression. Furthermore, Luman interacts with DC-STAMP, and controls its stability and localization. These results suggest that Luman regulates the multinucleation of osteoclasts by promoting cell fusion of mononuclear osteoclasts through DC-STAMP induction and intracellular distribution during osteoclastogenesis.

3.2427 α/β Hydrolase Domain-containing 6 (ABHD6) Degrades the Late Endosomal/Lysosomal Lipid Bis(monoacylglycero)phosphate

Pribasnig, M.A. et al
J. Biol. Chem., ***290(50)****, 29869-29881 (2015)*

α/β Hydrolase domain-containing 6 (ABHD6) can act as monoacylglycerol hydrolase and is believed to play a role in endocannabinoid signaling as well as in the pathogenesis of obesity and liver steatosis. However, the mechanistic link between gene function and disease is incompletely understood. Here we aimed to further characterize the role of ABHD6 in lipid metabolism. We show that mouse and human ABHD6 degrade bis(monoacylglycero)phosphate (BMP) with high specific activity. BMP, also known as lysobisphosphatidic acid, is enriched in late endosomes/lysosomes, where it plays a key role in the formation of intraluminal vesicles and in lipid sorting. Up to now, little has been known about the catabolism of this lipid. Our data demonstrate that ABHD6 is responsible for ~90% of the BMP hydrolase activity detected in the liver and that knockdown of ABHD6 increases hepatic BMP levels. Tissue fractionation and live-cell imaging experiments revealed that ABHD6 co-localizes with late endosomes/lysosomes. The enzyme is active at cytosolic pH and lacks acid hydrolase activity, implying that it degrades BMP exported from acidic organelles or *de novo*-formed BMP. In conclusion, our data suggest that ABHD6 controls BMP catabolism and is therefore part of the late endosomal/lysosomal lipid-sorting machinery.

3.2428 N-myristoyltransferase 1 interacts with calnexin at the endoplasmic reticulum
Dudek, E., Millott, R., Liu, W-X., Beauchamp, E., Berthiaume, L.G. and Michalak, M.
*Biochem. Biophys. Res. Comm., **468**, 889-893 (2015)*

Calnexin is a type 1 integral endoplasmic reticulum (ER) membrane molecular chaperone with a highly conserved C-terminal domain oriented to the cytoplasm. Protein *N*-myristoylation plays an important role in a wide variety of cellular signal transduction pathways and it is catalyzed by *N*-myristoyltransferase (NMT), a cytoplasmic and ER associated enzyme. Here using yeast two-hybrid screen, Western blot analysis, immunoprecipitation, immunolocalization and cellular fractionation we discovered that *N*-myristoyltransferase 1 interacts with calnexin at the ER. These observations point at a previously unrecognized contribution of calnexin to the retention of NMT1 at the ER membrane.

3.2429 Bacterial membrane vesicles, an overlooked environmental colloid: Biology, environmental perspectives and applications
Toyofuku, M., Tashiro, Y., Hasegawa, Y., Kurosawa, M. and Nomura, N.
*Advances in Colloid and Interface Science, **226**, 65-77 (2015)*

Phospholipid vesicles play important roles in biological systems. Bacteria are one of the most abundant organisms on Earth, and bacterial membrane vesicles (MVs) were first observed 50 years ago. Many bacteria release MVs to the environment that mainly consist of the cell membrane and typically range from 20 to 400 nm in size. Bacterial MVs are involved in several biological functions, such as delivery of cargo, virulence and gene transfer. MVs can be isolated from laboratory culture and directly from the environment, indicating their high abundance in and impact on ecosystems. Many colloidal particles in the environment ranging in size from 1 nm to 1 μm have been reported but not characterized at the molecular level, and MVs remain to be explored. Hence, MVs can be considered terra incognita in environmental colloid research. Although MV biogenesis and biological roles are yet to be fully understood, the accumulation of knowledge has opened new avenues for their applications. Via genetic engineering, the MV yield can be greatly increased, and the components of MVs can be tailored. Recent studies have demonstrated that MVs have promising potential for applications such as drug delivery systems and nanobiocatalysts. For instance, MV vaccines have been extensively studied and have already been approved in Europe. Recent MV studies have evoked great interest in the fields of biology and biotechnology, but fundamental questions, such as their transport in the environment or physicochemical features of MVs, remain to be addressed. In this review, we present the current understanding of bacterial MVs and environmental perspectives and further introduce their applications.

3.2430 Altered machinery of protein synthesis is region- and stage-dependent and is associated with α-synuclein oligomers in Parkinson's disease
Garcia-Esparcia, P., Hernandez-Ortega, K., Koneti, A., Gil, L., Delgado-Morales, R., Castano, E., Carmona, M. and Ferrer, I.
*Acta Neuropathologica Comm., **3**:76 (2015)*

Introduction
Parkinson's disease (PD) is characterized by the accumulation of abnormal α-synuclein in selected regions of the brain following a gradient of severity with disease progression. Whether this is accompanied by globally altered protein synthesis is poorly documented. The present study was carried out in PD stages 1-6 of Braak and middle-aged (MA) individuals without alterations in brain in the substantia nigra, frontal cortex area 8, angular gyrus, precuneus and putamen.
Results
Reduced mRNA expression of nucleolar proteins nucleolin (NCL), nucleophosmin (NPM1), nucleoplasmin 3 (NPM3) and upstream binding transcription factor (UBF), decreased NPM1 but not NPM3 nucleolar protein immunostaining in remaining neurons; diminished 18S rRNA, 28S rRNA; reduced expression of several mRNAs encoding ribosomal protein (RP) subunits; and altered protein levels of initiation factor eIF3 and elongation factor eEF2 of protein synthesis was found in the substantia nigra in PD along with disease progression. Although many of these changes can be related to neuron loss in the substantia nigra, selective alteration of certain factors indicates variable degree of vulnerability of mRNAs, rRNAs and proteins in degenerating sustantia nigra. NPM1 mRNA and 18S rRNA was increased in the frontal cortex area 8 at stage 5-6; modifications were less marked and region-dependent in the angular gyrus and precuneus. Several RPs were abnormally regulated in the frontal cortex area 8 and precuneus, but only one RP in the angular gyrus, in PD. Altered levels of eIF3 and eIF1, and decrease eEF1A and eEF2 protein levels were observed in the frontal cortex in PD. No modifications were found in the

putamen at any time of the study except transient modifications in 28S rRNA and only one RP mRNA at stages 5-6. These observations further indicate marked region-dependent and stage-dependent alterations in the cerebral cortex in PD. Altered solubility and α-synuclein oligomer formation, assessed in total homogenate fractions blotted with anti-α-synuclein oligomer-specific antibody, was demonstrated in the substantia nigra and frontal cortex, but not in the putamen, in PD. Dramatic increase in α-synuclein oligomers was also seen in fluorescent-activated cell sorter (FACS)-isolated nuclei in the frontal cortex in PD.

Conclusions

Altered machinery of protein synthesis is altered in the substantia nigra and cerebral cortex in PD being the frontal cortex area 8 more affected than the angular gyrus and precuneus; in contrast, pathways of protein synthesis are apparently preserved in the putamen. This is associated with the presence of α-synuclein oligomeric species in total homogenates; substantia nigra and frontal cortex are enriched, albeit with different band patterns, in α-synuclein oligomeric species, whereas α-synuclein oligomers are not detected in the putamen.

3.2431 Tannerella forsythia Outer Membrane Vesicles Are Enriched with Substrates of the Type IX Secretion System and TonB-Dependent Receptors

Veith, P.D., Chen, Y-Y., Chen, D., O'Brien-Simpson, N.M., Cecil, J., Holden, J.A., Lenzo, J.C. and Reynolds, E.C.

J. Proteome Res., ***14(12)***, *5355-5366 (2015)*

Tannerella forsythia, a Gram-negative oral bacterium closely associated with chronic periodontitis, naturally produces outer membrane vesicles (OMVs). In this study, OMVs were purified by gradient centrifugation, and the proteome was investigated together with cellular fractions using LC–MS/MS analyses of SDS-PAGE fractions, resulting in the identification of 872 proteins including 297 OMV proteins. Comparison of the OMV proteome with the subcellular proteomes led to the localization of 173 proteins to the vesicle membrane and 61 proteins to the vesicle lumen, while 27 substrates of the type IX secretion system were assigned to the vesicle surface. These substrates were generally enriched in OMVs; however, the stoichiometry of the S-layer proteins, TfsA and TfsB, was significantly altered, potentially to accommodate the higher curvature required of the S-layer around OMVs. A vast number of TonB-dependent receptors related to SusC, together with their associated SusD-like lipoproteins, were identified, and these were also relatively enriched in OMVs. In contrast, other lipoproteins were significantly depleted from the OMVs. This study identified the highest number of membrane-associated OMV proteins to date in any bacterium and conclusively demonstrates cargo sorting of particular classes of proteins, which may have significant impact on the virulence of OMVs.

3.2432 UNC-45A Is a Nonmuscle Myosin IIA Chaperone Required for NK Cell Cytotoxicity via Control of Lytic Granule Secretion

Iizuka, Y., Cichocki, F., Sieben, A., Sforza, F., karim, R., Coughlin, K., Isaksson Vogel, R., Gavioli, R., McCullar, V., Lenvik, T., Lee, M., Miller, J. and Bazzaro, M.

J. Immunol., ***195(10)***, *4760-4770 (2015)*

NK cell's killing is a tightly regulated process under the control of specific cytoskeletal proteins. This includes Wiskott–Aldrich syndrome protein, Wiskott–Aldrich syndrome protein–interacting protein, cofilin, Munc13-4, and nonmuscle myosin IIA (NMIIA). These proteins play a key role in controlling NK-mediated cytotoxicity either via regulating the attachment of lytic granules to the actin-based cytoskeleton or via promoting the cytoskeletal reorganization that is requisite for lytic granule release. UNC-45A is a highly conserved member of the UNC-45/CRO1/She4p family of proteins that act as chaperones for both conventional and nonconventional myosin. Although we and others have shown that in lower organisms and in mammalian cells NMIIA-associated functions, such as cytokinesis, cell motility, and organelle trafficking, are dependent upon the presence of UNC-45A, its role in NK-mediated functions is largely unknown. In this article, we describe UNC-45A as a key regulator of NK-mediated cell toxicity. Specifically we show that, in human NK cells, UNC-45A localize at the NK cell immunological synapse of activated NK cells and is part of the multiprotein complex formed during NK cell activation. Furthermore, we show that UNC-45A is disposable for NK cell immunological synapse formation and lytic granules reorientation but crucial for lytic granule exocytosis. Lastly, loss of UNC-45A leads to reduced NMIIA binding to actin, suggesting that UNC-45A is a crucial component in regulating human NK cell cytoskeletal dynamics via promoting the formation of actomyosin complexes.

3.2433 The COPII complex and lysosomal VAMP7 determine intracellular Salmonella localization and growth

Santos, J.C., Duchateau, M., Fredlund, J., Weiner, A., Mallet, A., Schmitt, C., matondo, M., Hourdel, V., Chamot-Rooke, J. and Enninga, J.
Cellular Microbiol., ***17(12)***, *1699-1720 (2015)*

Salmonella invades epithelial cells and survives within a membrane-bound compartment, the *Salmonella*-containing vacuole (SCV). We isolated and determined the host protein composition of the SCV at 30 min and 3 h of infection to identify and characterize novel regulators of intracellular bacterial localization and growth. Quantitation of the SCV protein content revealed 392 host proteins specifically enriched at SCVs, out of which 173 associated exclusively with early SCVs, 124 with maturing SCV and 95 proteins during both time-points. Vacuole interactions with endoplasmic reticulum-derived coat protein complex II vesicles modulate early steps of SCV maturation, promoting SCV rupture and bacterial hyper-replication within the host cytosol. On the other hand, SCV interactions with VAMP7-positive lysosome-like vesicles promote *Salmonella*-induced filament formation and bacterial growth within the late SCV. Our results reveal that the dynamic communication between the SCV and distinct host organelles affects both intracellular *Salmonella* localization and growth at successive steps of host cell invasion.

3.2434 The secret life of extracellular vesicles in metal homeostasis and neurodegeneration

Bellingham, S.A., Guo, B. and Hill, A.F.
Biol. Cell, ***107(11)***, *389-418 (2015)*

Biologically active metals such as copper, zinc and iron are fundamental for sustaining life in different organisms with the regulation of cellular metal homeostasis tightly controlled through proteins that coordinate metal uptake, efflux and detoxification. Many of the proteins involved in either uptake or efflux of metals are localised and function on the plasma membrane, traffic between intracellular compartments depending upon the cellular metal environment and can undergo recycling via the endosomal pathway. The biogenesis of exosomes also occurs within the endosomal system, with several major neurodegenerative disease proteins shown to be released in association with these vesicles, including the amyloid-β (Aβ) peptide in Alzheimer's disease and the infectious prion protein involved in Prion diseases. Aβ peptide and the prion protein also bind biologically active metals and are postulated to play important roles in metal homeostasis. In this review, we will discuss the role of extracellular vesicles in Alzheimer's and Prion diseases and explore their potential contribution to metal homeostasis.

3.2435 Redefining the Breast Cancer Exosome Proteome by Tandem Mass Tag Quantitative Proteomics and Multivariate Cluster Analysis

Clark, D.J., Fondrie, W.E., Liao, Z., Hanson, P.I., Fulton, A., Mao, L. and Yang, A.J.
Anal. Chem., ***87(20)***, *10462-10469 (2015)*

Exosomes are microvesicles of endocytic origin constitutively released by multiple cell types into the extracellular environment. With evidence that exosomes can be detected in the blood of patients with various malignancies, the development of a platform that uses exosomes as a diagnostic tool has been proposed. However, it has been difficult to truly define the exosome proteome due to the challenge of discerning contaminant proteins that may be identified via mass spectrometry using various exosome enrichment strategies. To better define the exosome proteome in breast cancer, we incorporated a combination of Tandem-Mass-Tag (TMT) quantitative proteomics approach and Support Vector Machine (SVM) cluster analysis of three conditioned media derived fractions corresponding to a 10 000*g* cellular debris pellet, a 100 000*g* crude exosome pellet, and an Optiprep enriched exosome pellet. The quantitative analysis identified 2 179 proteins in all three fractions, with known exosomal cargo proteins displaying at least a 2-fold enrichment in the exosome fraction based on the TMT protein ratios. Employing SVM cluster analysis allowed for the classification 251 proteins as “true” exosomal cargo proteins. This study provides a robust and vigorous framework for the future development of using exosomes as a potential multiprotein marker phenotyping tool that could be useful in breast cancer diagnosis and monitoring disease progression.

3.2436 In Vivo Differentiation of Therapeutic Insulin-Producing Cells from Bone Marrow Cells via Extracellular Vesicle-Mimetic Nanovesicles

Oh, K., Kim, S.R., Kim, D-K., Seo, M.W., Lee, C., Lee, H.M., Oh, J-E., Choi, E.Y., Lee, D-S., Gho, Y.S. and Park, K.S.
ACSNano, ***9(12)***, *11718-11727 (2015)*

The current diabetes mellitus pandemic constitutes an important global health problem. Reductions in the mass and function of β-cells contribute to most of the pathophysiology underlying diabetes. Thus, physiological control of blood glucose levels can be adequately restored by replacing functioning β-cell mass. Sources of functional islets for transplantation are limited, resulting in great interest in the development of alternate sources, and recent progress regarding cell fate change *via* utilization of extracellular vesicles, also known as exosomes and microvesicles, is notable. Thus, this study investigated the therapeutic capacity of extracellular vesicle-mimetic nanovesicles (NVs) derived from a murine pancreatic β-cell line. To differentiate insulin-producing cells effectively, a three-dimensional *in vivo* microenvironment was constructed in which extracellular vesicle-mimetic NVs were applied to subcutaneous Matrigel platforms containing bone marrow (BM) cells in diabetic immunocompromised mice. Long-term control of glucose levels was achieved over 60 days, and differentiation of donor BM cells into insulin-producing cells in the subcutaneous Matrigel platforms, which were composed of islet-like cell clusters with extensive capillary networks, was confirmed along with the expression of key pancreatic β-cell markers. The resectioning of the subcutaneous Matrigel platforms caused a rebound in blood glucose levels and confirmed the source of functioning β-cells. Thus, efficient differentiation of therapeutic insulin-producing cells was attained *in vivo* through the use of extracellular vesicle-mimetic NVs, which maintained physiological glucose levels.

Optimized exosome isolation protocol for cell culture supernatant and human plasma
Lobb, R.J., Becker, M., Wen, S.W., Wong, C.S.F., Wiegmans, A.P., Leimgruber, A. and Möller, A.
*J. Extracellular Vesicles, **4**:27031 (2015)*

Extracellular vesicles represent a rich source of novel biomarkers in the diagnosis and prognosis of disease. However, there is currently limited information elucidating the most efficient methods for obtaining high yields of pure exosomes, a subset of extracellular vesicles, from cell culture supernatant and complex biological fluids such as plasma. To this end, we comprehensively characterize a variety of exosome isolation protocols for their efficiency, yield and purity of isolated exosomes. Repeated ultracentrifugation steps can reduce the quality of exosome preparations leading to lower exosome yield. We show that concentration of cell culture conditioned media using ultrafiltration devices results in increased vesicle isolation when compared to traditional ultracentrifugation protocols. However, our data on using conditioned media isolated from the Non-Small-Cell Lung Cancer (NSCLC) SK-MES-1 cell line demonstrates that the choice of concentrating device can greatly impact the yield of isolated exosomes. We find that centrifuge-based concentrating methods are more appropriate than pressure-driven concentrating devices and allow the rapid isolation of exosomes from both NSCLC cell culture conditioned media and complex biological fluids. In fact to date, no protocol detailing exosome isolation utilizing current commercial methods from both cells and patient samples has been described. Utilizing tunable resistive pulse sensing and protein analysis, we provide a comparative analysis of 4 exosome isolation techniques, indicating their efficacy and preparation purity. Our results demonstrate that current precipitation protocols for the isolation of exosomes from cell culture conditioned media and plasma provide the least pure preparations of exosomes, whereas size exclusion isolation is comparable to density gradient purification of exosomes. We have identified current shortcomings in common extracellular vesicle isolation methods and provide a potential standardized method that is effective, reproducible and can be utilized for various starting materials. We believe this method will have extensive application in the growing field of extracellular vesicle research.

iSRAP – a one-touch research tool for rapid profiling of small RNA-seq data
Quek, C., Jung, C-h., Bellingham, S.A., Lonie, A. and Hill, A.F.
*J. Extracellular Vesicles, **4**:29454 (2015)*

Small non-coding RNAs have been significantly recognized as the key modulators in many biological processes, and are emerging as promising biomarkers for several diseases. These RNA species are transcribed in cells and can be packaged in extracellular vesicles, which are small vesicles released from many biotypes, and are involved in intercellular communication. Currently, the advent of next-generation sequencing (NGS) technology for high-throughput profiling has further advanced the biological insights of non-coding RNA on a genome-wide scale and has become the preferred approach for the discovery and quantification of non-coding RNA species. Despite the routine practice of NGS, the processing of large data sets poses difficulty for analysis before conducting downstream experiments. Often, the current analysis tools are designed for specific RNA species, such as microRNA, and are limited in flexibility for modifying parameters for optimization. An analysis tool that allows for maximum control of different

software is essential for drawing concrete conclusions for differentially expressed transcripts. Here, we developed a one-touch integrated small RNA analysis pipeline (iSRAP) research tool that is composed of widely used tools for rapid profiling of small RNAs. The performance test of iSRAP using publicly and in-house available data sets shows its ability of comprehensive profiling of small RNAs of various classes, and analysis of differentially expressed small RNAs. iSRAP offers comprehensive analysis of small RNA sequencing data that leverage informed decisions on the downstream analyses of small RNA studies, including extracellular vesicles such as exosomes.

3.2437 Extracellular vesicles secreted by Schistosoma mansoni contain protein vaccine candidates
Sotillo, J., Pearson, M., Potriquet, J., Becker, L., Pickering, D., Mulvenna, J. and Loukas, A.
Int. J. Parasitol., ***46***, *1-5 (2016)*

Herein we show for the first time that *Schistosoma mansoni* adult worms secrete exosome-like extracellular vesicles ranging from 50 to 130 nm in size. Extracellular vesicles were collected from the excretory/secretory products of cultured adult flukes and purified by Optiprep density gradient, resulting in highly pure extracellular vesicle preparations as confirmed by transmission electron microscopy and Nanosight tracking analysis. Extracellular vesicle proteomic analysis showed numerous known vaccine candidates, potential virulence factors and molecules implicated in feeding. These findings provide new avenues for the exploration of host–schistosome interactions and offer a potential mechanism by which some vaccine antigens exert their protective efficacy.

3.2438 Effect of exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation
Yamashita, T., Takahashi, Y., Nishiwaka, M. and Takakura, Y.
Eur. J. Pharmaceutics and Biopharmaceutics, ***98***, *1-8 (2016)*

Exosomes, which are expected to be delivery systems for biomolecules such as nucleic acids, are collected by several methods. However, the effect of exosome isolation methods on the characteristics of exosomes as drug carriers, such as recovery efficiency after sterile filtration and pharmacokinetics, has not been investigated despite the importance of these characteristics for the development of exosome-based delivery systems. In the present study, exosomes collected from murine melanoma B16-BL6 cells by several methods were compared with respect to dispersibility, recovery rate after filtering, and clearance from the blood circulation in mice. The exosomes were collected by three ultracentrifugation-based methods: simple ultracentrifugation/pelleting (pelleting method), ultracentrifugation with an iodixanol cushion (cushion method), and ultracentrifugation on an iodixanol density gradient (gradient method). The isolation methods had little effect on the particle number of exosomes. In contrast, transmission electron microscopy observation and size distribution measurement using tunable resistive pulse sensing indicated that the exosomes of the gradient method were more dispersed than the others. The exosomes were labeled with *Gaussia* luciferase and intravenously injected into mice. Clearance of injected exosomes from the blood circulation did not significantly change with isolation methods. When the exosomes were filtered using a 0.2-μm filter, the recovery rate was 82% for the exosomes of the gradient method, whereas it was less than 50% for the others. These results indicate that the exosome isolation method markedly affects the dispersibility and filtration efficiency of the exosomes.

3.2439 βA3/A1-crystallin and persistent fetal vasculature (PFV) disease of the eye
Ziegler, Jr., J.S., Valapala, M., Shang, P., Hose, S., Goldberg, M.F. and Sinha, D.
Biochim. Biophys. Acta, ***1860***, *287-298 (2016)*

Background
Persistent fetal vasculature (PFV) is a human disease in which the fetal vasculature of the eye fails to regress normally. The fetal, or hyaloid, vasculature nourishes the lens and retina during ocular development, subsequently regressing after formation of the retinal vessels. PFV causes serious congenital pathologies and is responsible for as much as 5% of blindness in the United States.
Scope of review
The causes of PFV are poorly understood, however there are a number of animal models in which aspects of the disease are present. One such model results from mutation or elimination of the gene (*Cryba1*) encoding βA3/A1-crystallin. In this review we focus on the possible mechanisms whereby loss of functional βA3/A1-crystallin might lead to PFV.
Major conclusions

Cryba1 is abundantly expressed in the lens, but is also expressed in certain other ocular cells, including astrocytes. In animal models lacking βA3/A1-crystallin, astrocyte numbers are increased and they migrate abnormally from the retina to ensheath the persistent hyaloid artery. Evidence is presented that the absence of functional βA3/A1-crystallin causes failure of the normal acidification of endolysosomal compartments in the astrocytes, leading to impairment of certain critical signaling pathways, including mTOR and Notch/STAT3.

3.2440 A fluorescent cholesterol analogue for observation of free cholesterol in the plasma membrane of live cells

Ogawa, Y. and Tanaka, M.
*Anal. Biochem., **492**, 49-55 (2016)*

Free cholesterol in mammalian cells resides mostly in the plasma membrane, where it plays an important role in cellular homeostasis. We synthesized a new fluorescent cholesterol analogue that retained an intact alkyl chain and the sterane backbone of cholesterol. The hydroxyl group of cholesterol was converted into an amino group that was covalently linked to the fluorophore tetramethylrhodamine to retain the ability to form hydrogen bonds with adjacent molecules. Incubating live MDCK (Madin–Darby canine kidney) cells with our fluorescent cholesterol analogue resulted in the generation of intense signals that were detected by microscopy at the plasma membrane. Incubation with the analogue exerted minimal, if any, influence on cell growth, indicating that it could serve as a useful tool for analyzing free cholesterol at the plasma membrane.

3.2441 Identification of EDIL3 on extracellular vesicles involved in breast cancer cell invasion

Lee, J-E., Moon, P-G., Cho, Y-E., Kim, Y-B., Kim, I-S., park, H. and Baek, M-C.
*J. Proteomics, **131**, 17-28 (2016)*

Cancer cell-derived extracellular vesicles have been linked to the pathogenesis of various cancers; however, the role of extracellular vesicles in tumorigenesis remains unclear. To identify extracellular vesicle proteins involved in cancer metastasis, quantitative proteomic analyses were performed on extracellular vesicles derived from two representative breast cancer cell lines: the less invasive MCF-7 and the invasive MDA-MB-231. Proteomic analysis allowed for the identification of 270 proteins in the extracellular vesicles. Here we report a new function of EDIL3 on extracellular vesicles, which are sufficient for enhancement of cell invasion and for acceleration of lung metastasis *in vivo*. This invasion is most likely mediated via the integrin-FAK signaling cascade in breast cancer cells. However, these effects are suppressed when EDIL3 is inactivated, providing evidence for a critical role of EDIL3 in development of cancer. Consistently, in human patients with metastatic breast cancer, the levels of EDIL3 on circulating extracellular vesicles are significantly elevated. This information is a remarkable breakthrough in understanding of the molecular mechanism underlying metastasis of breast cancer as well as in the research for cancer biomarkers using circulating extracellular vesicles. Furthermore, targeting EDIL3 on extracellular vesicles may lead to a new therapeutic option for treatment of breast cancer.

3.2442 Bovine milk-derived exosomes for drug delivery

Munagala, R., Aqil, F., Jeybalan, J. and Gupta, R.
*Cancer Lett., **371**, 48-61 (2016)*

Exosomes are biological nanovesicles that are involved in cell–cell communication via the functionally-active cargo (such as miRNA, mRNA, DNA and proteins). Because of their nanosize, exosomes are explored as nanodevices for the development of new therapeutic applications. However, bulk, safe and cost-effective production of exosomes is not available. Here, we show that bovine milk can serve as a scalable source of exosomes that can act as a carrier for chemotherapeutic/chemopreventive agents. Drug-loaded exosomes showed significantly higher efficacy compared to free drug in cell culture studies and against lung tumor xenografts *in vivo*. Moreover, tumor targeting ligands such as folate increased cancer-cell targeting of the exosomes resulting in enhanced tumor reduction. Milk exosomes exhibited cross-species tolerance with no adverse immune and inflammatory response. Thus, we show the versatility of milk exosomes with respect to the cargo it can carry and ability to achieve tumor targetability. This is the first report to identify a biocompatible and cost-effective means of exosomes to enhance oral bioavailability, improve efficacy and safety of drugs.

3.2443 Peroxisomal Pex11 is a pore-forming protein homologous to TRPM channels

Mindthoff, S., Grunau, S., Steinfort, L.L., Girzalsky, W., Hiltunen, J.K., Erdmann, R. and Antonenkov, V.D.

*Biochim. Biophys. Acta, **1863**, 271-283 (2016)*

More than 30 proteins (Pex proteins) are known to participate in the biogenesis of peroxisomes—ubiquitous oxidative organelles involved in lipid and ROS metabolism. The Pex11 family of homologous proteins is responsible for division and proliferation of peroxisomes. We show that yeast Pex11 is a pore-forming protein sharing sequence similarity with TRPM cation-selective channels. The Pex11 channel with a conductance of Λ = 4.1 nS in 1.0 M KCl is moderately cation-selective (P_{K^+}/P_{Cl^-} = 1.85) and resistant to voltage-dependent closing. The estimated size of the channel's pore (r ~ 0.6 nm) supports the notion that Pex11 conducts solutes with molecular mass below 300–400 Da. We localized the channel's selectivity determining sequence. Overexpression of Pex11 resulted in acceleration of fatty acids β-oxidation in intact cells but not in the corresponding lysates. The β-oxidation was affected in cells by expression of the Pex11 protein carrying point mutations in the selectivity determining sequence. These data suggest that the Pex11-dependent transmembrane traffic of metabolites may be a rate-limiting step in the β-oxidation of fatty acids. This conclusion was corroborated by analysis of the rate of β-oxidation in yeast strains expressing Pex11 with mutations mimicking constitutively phosphorylated (S165D, S167D) or unphosphorylated (S165A, S167A) protein. The results suggest that phosphorylation of Pex11 is a mechanism that can control the peroxisomal β-oxidation rate.
Our results disclose an unexpected function of Pex11 as a non-selective channel responsible for transfer of metabolites across peroxisomal membrane. The data indicate that peroxins may be involved in peroxisomal metabolic processes in addition to their role in peroxisome biogenesis.

3.2444 ABCA1-dependent sterol release: sterol molecule specificity and potential membrane domain for HDL biogenesis

Yamauchi, Y., Yokoyama, S. and Chang, T-Y.

*J. Lipid Res., **57**, 77-88 (2016)*

Mammalian cells synthesize various sterol molecules, including the C30 sterol, lanosterol, as cholesterol precursors in the endoplasmic reticulum. The build-up of precursor sterols, including lanosterol, displays cellular toxicity. Precursor sterols are found in plasma HDL. How these structurally different sterols are released from cells is poorly understood. Here, we show that newly synthesized precursor sterols arriving at the plasma membrane (PM) are removed by extracellular apoA-I in a manner dependent on ABCA1, a key macromolecule for HDL biogenesis. Analysis of sterol molecules by GC-MS and tracing the fate of radiolabeled acetate-derived sterols in normal and mutant Niemann-Pick type C cells reveal that ABCA1 prefers newly synthesized sterols, especially lanosterol, as the substrates before they are internalized from the PM. We also show that ABCA1 resides in a cholesterol-rich membrane domain resistant to the mild detergent, Brij 98. Blocking ACAT activity increases the cholesterol contents of this domain. Newly synthesized C29/C30 sterols are transiently enriched within this domain, but rapidly disappear from this domain with a half-life of less than 1 h. Our work shows that substantial amounts of precursor sterols are transported to a certain PM domain and are removed by the ABCA1-dependent pathway.

3.2445 A draft map of the mouse pluripotent stem cell spatial proteome

Christoforou, A., Mulvey, C.M., Breckels, L.M., Geladaki, A., Hurrell, T., Hayward, P.C., Naake, T., Gatto, L., Viner, R., Martinez Arias, A. and Lilley, K.S.

*Nature Communications, **7**:8992 (2016)*

Knowledge of the subcellular distribution of proteins is vital for understanding cellular mechanisms. Capturing the subcellular proteome in a single experiment has proven challenging, with studies focusing on specific compartments or assigning proteins to subcellular niches with low resolution and/or accuracy. Here we introduce hyperLOPIT, a method that couples extensive fractionation, quantitative high-resolution accurate mass spectrometry with multivariate data analysis. We apply hyperLOPIT to a pluripotent stem cell population whose subcellular proteome has not been extensively studied. We provide localization data on over 5,000 proteins with unprecedented spatial resolution to reveal the organization of organelles, sub-organellar compartments, protein complexes, functional networks and steady-state dynamics of proteins and unexpected subcellular locations. The method paves the way for characterizing the impact of post-transcriptional and post-translational modification on protein location and studies involving proteome-level locational changes on cellular perturbation. An interactive open-source resource is presented that enables exploration of these data.

3.2446 **The proprotein convertase PC1/3 regulates TLR9 trafficking and the associated signaling pathways**
Duhamel, M., Rodet, F., Murgoci, A.N., Desjardins, r., Gagnon, H., wisztorsski, M., Fournier, I., day, R. and Salzet, M.
*Scientific Reports, **6**:19360 (2016)*

Endosomal TLR9 is considered as a potent anti-tumoral therapeutic target. Therefore, it is crucial to decipher the mechanisms controlling its trafficking since it determines TLR9 activation and signalling. At present, the scarcity of molecular information regarding the control of this trafficking and signalling is noticeable. We have recently demonstrated that in macrophages, proprotein convertase 1/3 (PC1/3) is a key regulator of TLR4 Myd88-dependent signalling. In the present study, we established that PC1/3 also regulates the endosomal TLR9. Under CpG-ODN challenge, we found that PC1/3 traffics rapidly to co-localize with TLR9 in CpG-ODN-containing endosomes with acidic pH. In PC1/3 knockdown macrophages, compartmentalization of TLR9 was altered and TLR9 clustered in multivesicular bodies (MVB) as demonstrated by co-localization with Rab7. This demonstrates that PC1/3 controls TLR9 trafficking. This clustering of TLR9 in MVB dampened the anti-inflammatory STAT3 signalling pathway while it promoted the pro-inflammatory NF-kB pathway. As a result, macrophages from PC1/3 KO mice and rat PC1/3-KD NR8383 macrophages secreted more pro-inflammatory cytokines such as TNF-α, IL6, IL1α and CXCL2. This is indicative of a M1 pro-inflammatory phenotype. Therefore, PC1/3 KD macrophages represent a relevant mean for cell therapy as “Trojan” macrophages.

3.2447 **Exosomes Mediate LTB4 Release during Neutrophil Chemotaxis**
Majumdar, R., Tameh, A.T. and Parent, C.A.
*PloS Biology, **14(1)**, e1002336 (2016)*

Leukotriene B_4 (LTB_4) is secreted by chemotactic neutrophils, forming a secondary gradient that amplifies the reach of primary chemoattractants. This strategy increases the recruitment range for neutrophils and is important during inflammation. Here, we show that LTB_4 and its synthesizing enzymes localize to intracellular multivesicular bodies that, upon stimulation, release their content as exosomes. Purified exosomes can activate resting neutrophils and elicit chemotactic activity in a LTB_4 receptor-dependent manner. Inhibition of exosome release leads to loss of directional motility with concomitant loss of LTB_4 release. Our findings establish that the exosomal pool of LTB_4 acts in an autocrine fashion to sensitize neutrophils towards the primary chemoattractant, and in a paracrine fashion to mediate the recruitment of neighboring neutrophils in trans. We envision that this mechanism is used by other signals to foster communication between cells in harsh extracellular environments.

3.2448 **Modulation of Mitochondrial Antiviral Signaling by Human Herpesvirus 8 Interferon Regulatory Factor 1**
Hwang, K.Y. and Choi, Y.B.
*J. Virol., **90(1)**, 506-520 (2016)*

Mitochondrial lipid raft-like microdomains, experimentally also termed mitochondrial detergent-resistant membrane fractions (mDRM), play a role as platforms for recruiting signaling molecules involved in antiviral responses such as apoptosis and innate immunity. Viruses can modulate mitochondrial functions for their own survival and replication. However, viral regulation of the antiviral responses via mDRM remains incompletely understood. Here, we report that human herpesvirus 8 (HHV-8) gene product viral interferon regulatory factor 1 (vIRF-1) is targeted to mDRM during virus replication and negatively regulates the mitochondrial antiviral signaling protein (MAVS)-mediated antiviral responses. The N-terminal region of vIRF-1 interacts directly with membrane lipids, including cardiolipin. In addition, a GxRP motif within the N terminus of vIRF-1, conserved in the mDRM-targeting region of mitochondrial proteins, including PTEN-induced putative kinase 1 (PINK1) and MAVS, was found to be important for vIRF-1 association with mitochondria. Furthermore, MAVS, which has the potential to promote vIRF-1 targeting to mDRM possibly by inducing cardiolipin exposure on the outer membrane of mitochondria, interacts with vIRF-1, which, in turn, inhibits MAVS-mediated antiviral signaling. Consistent with these results, vIRF-1 targeting to mDRM contributes to promotion of HHV-8 productive replication and inhibition of associated apoptosis. Combined, our results suggest novel molecular mechanisms for negative-feedback regulation of MAVS by vIRF-1 during virus replication.

3.2449 PTP-PEST controls EphA3 activation and ephrin-induced cytoskeletal remodeling
Mansour, M., Nievergali, E., gegenbauer, K., Llerena, C., Atapattu, L., Halle, M., Tremblay, M.L., Janes, P.W. and lackmann, M.
J. Cell Sci., ***129****, 277-289 (2016)*

Eph receptors and their corresponding membrane-bound ephrin ligands regulate cell positioning and establish tissue patterns during embryonic and oncogenic development. Emerging evidence suggests that assembly of polymeric Eph signalling clusters relies on cytoskeletal reorganisation and underlies regulation by protein tyrosine phosphatases (PTPs). PTP-PEST (also known as PTPN12) is a central regulator of actin cytoskeletal dynamics. Here, we demonstrate that an N-terminal fragment of PTP-PEST, generated through an ephrinA5-triggered and spatially confined cleavage mediated by caspase-3, attenuates EphA3 receptor activation and its internalisation. Isolation of EphA3 receptor signalling clusters within intact plasma membrane fragments obtained by detergent-free cell fractionation reveals that stimulation of cells with ephrin triggers effective recruitment of this catalytically active truncated form of PTP-PEST together with key cytoskeletal and focal adhesion proteins. Importantly, modulation of actin polymerisation using pharmacological and dominant-negative approaches affects EphA3 phosphorylation in a similar manner to overexpression of PTP-PEST. We conclude that PTP-PEST regulates EphA3 activation both by affecting cytoskeletal remodelling and through its direct action as a PTP controlling EphA3 phosphorylation, indicating its multifaceted regulation of Eph signalling.

3.2450 Lysosomes serve as a platform for hepatitis A virus particle maturation and nonlytic release
Seggewiss, N., Paulmann, D. and Dotzauer, A.
Arch. Virol., ***161(1)****, 43-52 (2016)*

Early studies on hepatitis A virus (HAV) in cell culture demonstrated the inclusion of several viral particles in an intracellular lipid-bilayer membrane. However, the origin of these virus-associated membranes and the mechanism for the non-lytic release of HAV into bile are still unknown. Analyzing the association of this virus with cell organelles, we found that newly synthesized HAV particles accumulate in lysosomal organelles and that lysosomal enzymes are involved in the maturation cleavage of the virion. Furthermore, by inhibiting the processes of fusion of lysosomes with the plasma membrane, we found that the nonlytic release of HAV from infected cells occurs via lysosome-related organelles.

3.2451 Borrelia burgdorferi HtrA: evidence for twofold proteolysis of outer membrane protein p66
Coleman, J.L., Toledo, A. and Benach, J.L.
Mol. Microbiol., ***99(1)****, 135-150 (2016)*

In prokaryotes, members of the High Temperature Requirement A (HtrA) family of serine proteases function in the periplasm to degrade damaged or improperly folded membrane proteins. *Borrelia burgdorferi*, the agent of Lyme disease, codes for a single HtrA homolog. Two-dimensional electrophoresis analysis of *B. burgdorferi* B31A3 and a strain that overexpresses HtrA (A3HtrAOE) identified a downregulated protein in A3HtrAOE with a mass, pI and MALDI-TOF spectrum consistent with outer membrane protein p66. P66 and HtrA from cellular lysates partitioned into detergent-resistant membranes, which contain cholesterol-glycolipid-rich membrane regions known as lipid rafts, suggesting that HtrA and p66 may reside together in lipid rafts also. This agrees with previous work from our laboratory, which showed that HtrA and p66 are constituents of *B. burgdorferi* outer membrane vesicles. HtrA degraded p66 *in vitro* and A3HtrAOE expressed reduced levels of p66 *in vivo*. Fluorescence confocal microscopy revealed that HtrA and p66 colocalize in the membrane. The association of HtrA and p66 establishes that they could interact efficiently and their protease/substrate relationship provides functional relevance to this interaction. A3HtrAOE also showed reduced levels of p66 transcript in comparison with wild-type B31A3, indicating that HtrA-mediated regulation of p66 may occur at multiple levels.

3.2452 Targeting Viral Proteostasis Limits Influenza Virus, HIV, and Dengue Virus Infection
Heaton, N. et al
Immunity, ***44(1)****, 46-58 (2016)*

Viruses are obligate parasites and thus require the machinery of the host cell to replicate. Inhibition of host factors co-opted during active infection is a strategy hosts use to suppress viral replication and a potential pan-antiviral therapy. To define the cellular proteins and processes required for a virus during infection is thus crucial to understanding the mechanisms of virally induced disease. In this report, we generated fully infectious tagged influenza viruses and used infection-based proteomics to identify pivotal arms of cellular

signaling required for influenza virus growth and infectivity. Using mathematical modeling and genetic and pharmacologic approaches, we revealed that modulation of Sec61-mediated cotranslational translocation selectively impaired glycoprotein proteostasis of influenza as well as HIV and dengue viruses and led to inhibition of viral growth and infectivity. Thus, by studying virus-human protein-protein interactions in the context of active replication, we have identified targetable host factors for broad-spectrum antiviral therapies.

3.2453 Exosomes from HIV-1-infected Cells Stimulate Production of Pro-inflammatory Cytokines through Trans-activating Response (TAR) RNA
Sampey, G.C., Saifuddin, M., Schwab, A., Barclay, r., Punya, S., Chung, M-C., Hakami, R.M., Zadeh, M.A., Lepene, B., Klase, Z.A., El-Hage, N., Young, M., Iordanskiy, S. and kashanchi, F.
J. Biol. Chem., ***291(3)***, *1251-1266 (2016)*

HIV-1 infection results in a chronic illness because long-term highly active antiretroviral therapy can lower viral titers to an undetectable level. However, discontinuation of therapy rapidly increases virus burden. Moreover, patients under highly active antiretroviral therapy frequently develop various metabolic disorders, neurocognitive abnormalities, and cardiovascular diseases. We have previously shown that exosomes containing trans-activating response (TAR) element RNA enhance susceptibility of undifferentiated naive cells to HIV-1 infection. This study indicates that exosomes from HIV-1-infected primary cells are highly abundant with TAR RNA as detected by RT-real time PCR. Interestingly, up to a million copies of TAR RNA/μl were also detected in the serum from HIV-1-infected humanized mice suggesting that TAR RNA may be stable *in vivo*. Incubation of exosomes from HIV-1-infected cells with primary macrophages resulted in a dramatic increase of proinflammatory cytokines, IL-6 and TNF-β, indicating that exosomes containing TAR RNA could play a direct role in control of cytokine gene expression. The intact TAR molecule was able to bind to PKR and TLR3 effectively, whereas the 5′ and 3′ stems (TAR microRNAs) bound best to TLR7 and -8 and none to PKR. Binding of TAR to PKR did not result in its phosphorylation, and therefore, TAR may be a dominant negative decoy molecule in cells. The TLR binding through either TAR RNA or TAR microRNA potentially can activate the NF-κB pathway and regulate cytokine expression. Collectively, these results imply that exosomes containing TAR RNA could directly affect the proinflammatory cytokine gene expression and may explain a possible mechanism of inflammation observed in HIV-1-infected patients under cART.

3.2454 Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions
Purushothaman, A., Bandari, S.K., Liu, J., Mobley, J.A., Brown, E.E. and Sanderson, R.D.
J. Biol. Chem., ***291(4)***, *1652-1663 (2016)*

Exosomes regulate cell behavior by binding to and delivering their cargo to target cells; however, the mechanisms mediating exosome-cell interactions are poorly understood. Heparan sulfates on target cell surfaces can act as receptors for exosome uptake, but the ligand for heparan sulfate on exosomes has not been identified. Using exosomes isolated from myeloma cell lines and from myeloma patients, we identify exosomal fibronectin as a key heparan sulfate-binding ligand and mediator of exosome-cell interactions. We discovered that heparan sulfate plays a dual role in exosome-cell interaction; heparan sulfate on exosomes captures fibronectin, and on target cells it acts as a receptor for fibronectin. Removal of heparan sulfate from the exosome surface releases fibronectin and dramatically inhibits exosome-target cell interaction. Antibody specific for the Hep-II heparin-binding domain of fibronectin blocks exosome interaction with tumor cells or with marrow stromal cells. Regarding exosome function, fibronectin-mediated binding of exosomes to myeloma cells activated p38 and pERK signaling and expression of downstream target genes DKK1 and MMP-9, two molecules that promote myeloma progression. Antibody against fibronectin inhibited the ability of myeloma-derived exosomes to stimulate endothelial cell invasion. Heparin or heparin mimetics including Roneparstat, a modified heparin in phase I trials in myeloma patients, significantly inhibited exosome-cell interactions. These studies provide the first evidence that fibronectin binding to heparan sulfate mediates exosome-cell interactions, revealing a fundamental mechanism important for exosome-mediated cross-talk within tumor microenvironments. Moreover, these results imply that therapeutic disruption of fibronectin-heparan sulfate interactions will negatively impact myeloma tumor growth and progression.

3.2455 A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria
Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O:, Klug, L., Gadermeier, B., Weinzerl, K., Prassl, R., Lass, G., Daum. G., Reidl, J., Feldman, M.F. and Schild, S.
Nature Communications, ***7****:10515 (2016)*

Bacterial outer membrane vesicles (OMVs) have important biological roles in pathogenesis and intercellular interactions, but a general mechanism of OMV formation is lacking. Here we show that the VacJ/Yrb ABC (ATP-binding cassette) transport system, a proposed phospholipid transporter, is involved in OMV formation. Deletion or repression of VacJ/Yrb increases OMV production in two distantly related Gram-negative bacteria, *Haemophilus influenzae* and *Vibrio cholerae*. Lipidome analyses demonstrate that OMVs from VacJ/Yrb-defective mutants in *H. influenzae* are enriched in phospholipids and certain fatty acids. Furthermore, we demonstrate that OMV production and regulation of the VacJ/Yrb ABC transport system respond to iron starvation. Our results suggest a new general mechanism of OMV biogenesis based on phospholipid accumulation in the outer leaflet of the outer membrane. This mechanism is highly conserved among Gram-negative bacteria, provides a means for regulation, can account for OMV formation under all growth conditions, and might have important pathophysiological roles *in vivo*.

3.2456 H2AX phosphorylation and DNA damage kinase activity are dispensable for herpes simplex virus replication

Botting, C., Lu, X. and Triezenberg, S.J.
Virology J., ***13****:15 (2016)*

Background
Herpes simplex virus type 1 (HSV-1) can establish both lytic and latent infections in humans. The phosphorylation of histone H2AX, a common marker of DNA damage, during lytic infection by HSV-1 is well established. However, the role(s) of H2AX phosphorylation in lytic infection remain unclear.
Methods
Following infection of human foreskin fibroblasts by HSV-1 or HSV-2, we assayed the phosphorylation of H2AX in the presence of inhibitors of transcription, translation, or viral DNA replication, or in the presence of inhibitors of ATM and ATR kinases (KU-55933 and VE-821, respectively). We also assayed viral replication in fibroblasts in the presence of the kinase inhibitors or siRNAs specific for ATM and ATR, as well as in cell lines deficient for either ATR or ATM.
Results
The expression of viral immediate-early and early proteins (including the viral DNA polymerase), but not viral DNA replication or late protein expression, were required for H2AX phosphorylation following HSV-1 infection. Inhibition of ATM kinase activity prevented HSV-stimulated H2AX phosphorylation but had only a minor effect on DNA replication and virus yield in HFF cells. These results differ from previous reports of a dramatic reduction in viral yield following chemical inhibition of ATM in oral keratinocytes or following infection of $ATM^{-/-}$ cells. Inhibition of the closely related kinase ATR (whether by chemical inhibitor or siRNA disruption) had no effect on H2AX phosphorylation and reduced viral DNA replication only moderately. During infection by HSV-2, H2AX phosphorylation was similarly dispensable but was dependent on both ATM activity and viral DNA replication.
Conclusion
H2AX phosphorylation represents a cell type-specific and virus type-specific host response to HSV infection with little impact on viral infection.

3.2457 Iodixanol Gradient Centrifugation to Separate Components of the Low-Density Membrane Fraction from 3T3-L1 Adipocytes

Sadler, J.B.A., lamb, C.A., Gould, G.W. and Bryant, N.J.
Cold Spring Harbor Protoc., ***pdb prot083709*** *(2016)*

We optimized a set of fractionation techniques to facilitate the isolation of subcellular compartments containing insulin-sensitive glucose transporter isoform 4 (GLUT4), which is mobilized from GLUT4 storage vesicles (GSVs) in fat and muscle cells in response to insulin. In the absence of insulin, GLUT4 undergoes a continuous cycle of GSV formation and fusion with other compartments. Full membrane fractionation of 3T3-L1 adipocytes produces a low-density membrane fraction that contains both the constitutive recycling pool (the endosomal recycling compartments) and the insulin-sensitive pool (the GSVs). These two pools can be separated based on density using iodixanol gradient centrifugation, described here.

3.2458 Triple SILAC quantitative proteomic analysis reveals differential abundance of cell signaling proteins between normal and lung cancer-derived exosomes

Clark, D.J., Fondrie, W.E., Yang, A. and Mao, L.

*J. Proteomics, **133**, 161-169 (2016)*

Exosomes are 30–100 nm sized membrane vesicles released by cells into the extracellular space that mediate intercellular communication via transfer of proteins and other biological molecules. To better understand the role of these microvesicles in lung carcinogenesis, we employed a Triple SILAC quantitative proteomic strategy to examine the differential protein abundance between exosomes derived from an immortalized normal bronchial epithelial cell line and two non-small cell lung cancer (NSCLC) cell lines harboring distinct activating mutations in the cell signaling molecules: Kirsten rat sarcoma viral oncogene homolog (KRAS) or epidermal growth factor receptor (EGFR). In total, we were able to quantify 721 exosomal proteins derived from the three cell lines. Proteins associated with signal transduction, including EGFR, GRB2 and SRC, were enriched in NSCLC exosomes, and could actively regulate cell proliferation in recipient cells. This study's investigation of the NSCLC exosomal proteome has identified enriched protein cargo that can contribute to lung cancer progression, which may have potential clinical implications in biomarker development for patients with NSCLC.

3.2459 Exosome-mediated inflammasome signaling after central nervous system injury
De Rivero Vaccari, J.P., Brand III, F., Adamczak, S., Lee, S.W., Perez-Barcena, J., Wang, M.Y., Bullock, M.R., Dalton Dietrich, W. and Keane, R.W.
*J. Neurochem., **136(S1)**, 39-48 82016)*

Neuroinflammation is a response against harmful effects of diverse stimuli and participates in the pathogenesis of brain and spinal cord injury (SCI). The innate immune response plays a role in neuroinflammation following CNS injury via activation of multiprotein complexes termed inflammasomes that regulate the activation of caspase 1 and the processing of the pro-inflammatory cytokines IL-1β and IL-18. We report here that the expression of components of the nucleotide-binding and oligomerization domain (NOD)-like receptor protein-1 (NLRP-1) inflammasome, apoptosis speck-like protein containing a caspase recruitment domain (ASC), and caspase 1 are significantly elevated in spinal cord motor neurons and cortical neurons after CNS trauma. Moreover, NLRP1 inflammasome proteins are present in exosomes derived from CSF of SCI and traumatic brain-injured patients following trauma. To investigate whether exosomes could be used to therapeutically block inflammasome activation in the CNS, exosomes were isolated from embryonic cortical neuronal cultures and loaded with short-interfering RNA (siRNA) against ASC and administered to spinal cord-injured animals. Neuronal-derived exosomes crossed the injured blood–spinal cord barrier, and delivered their cargo *in vivo*, resulting in knockdown of ASC protein levels by approximately 76% when compared to SCI rats treated with scrambled siRNA. Surprisingly, siRNA silencing of ASC also led to a significant decrease in caspase 1 activation and processing of IL-1β after SCI. These findings indicate that exosome-mediated siRNA delivery may be a strong candidate to block inflammasome activation following CNS injury.
We propose the following signaling cascade for inflammasome activation in peripheral tissues after CNS injury: CNS trauma induces inflammasome activation in the nervous system and secretion of exosomes containing inflammasome protein cargo into cerebral spinal fluid. The inflammasome containing exosomes then fuse with target cells to activate the innate immune response in peripheral tissues. We suggest that these findings may be used to develop new therapeutics to treat the devastating inflammation and cell destruction evoked by CNS injuries. IL-1β and IL-18 = pro-inflammatory cytokines.

3.2460 The influence of a caveolin-1 mutant on the function of P-glycoprotein
Lee, C-Y., Lai, T-Y., Tsai, M-K., Ou-Yang, P., Tsasi, C-Y., Wu, S-W., Hsu, L-C. and Chen, J-S.
*Scientific Reports, **6**:20486 (2016)*

The genetic heterogeneity in cancer cells has an increased chance in the acquisition of new mutant such as drug-resistant phenotype in cancer cells. The phenotype of drug resistance in cancer cells could be evaluated by the number or function of drug transporters on cell membranes, which would lead to decreased intracellular anti-cancer drugs concentration. Caveolae are flask-shaped invaginations on cell membrane that function in membrane trafficking, endocytosis, and as a compartment where receptors and signaling proteins are concentrated. Caveolin-1 (CAV1) is the principal structural protein of caveolae and closely correlates with multidrug resistance in cancer cells. In a systematic study of the ubiquitin-modified proteome, lysine 176 of CAV1 was identified as a potential post-translational modification site for ubiquitination. In this article, we identified a mutation at lysine 176 to arginine (K176R) on CAV1 would interfere with the biogenesis of caveolae and broke the interaction of CAV1 with P-glycoprotein. Functional assays further revealed that K176R mutant of CAV1 in cancer cells increased the transport

activity of P-glycoprotein and decreased the killing ability of anti-cancer drugs in non-small-cell lung cancer cell lines.

3.2461 Exosomes as new diagnostic tools in CNS diseases
Kanninen, K.M., Bister, N., Koistinaho, J. and Malm, T.
*Biochim. Biophys. Acta, **1862**, 403-410 (2016)*

Exosomes are small extracellular vesicles that modulate important functions in physiology and under pathological conditions of the central nervous system (CNS). Exosomal contents, proteins, lipids and various RNA species, are altered during disease. The fact that exosomes are released into the blood stream from blood cells and endothelial cells responding to CNS diseases as well as from the brain and spinal cord, and that they express markers which allow their tracking to the cell of origin, makes the use of exosomes for diagnostic purposes and biomarker discovery particularly appealing. While the utilization of exosomes for diagnostics in diseases affecting the CNS are still in the early stages of discovery and development, it is expected that through further research and fervent development of protocols relating to isolation and purification the true potential of exosomes derived from the CNS will be harnessed for more effective clinical disease diagnosis. In this review we begin with a short introduction to the origin, composition and function of exosomes in the CNS. Next we discuss the current status of methodologies related to isolation and detection of CNS exosomes. We end with an account of exosomes in diagnostics and biomarker discovery, which focuses on three diseases of the CNS: Alzheimer's disease, multiple sclerosis, and stroke. This article is part of a Special Issue entitled: Neuro Inflammation edited by Helga E. de Vries and Markus Schwaninger.

3.2462 Prerequisites for the analysis and sorting of extracellular vesicle subpopulations by high-resolution flow cytometry
Kormelink, T.G., Arkesteijn, G.J.A., nauwelaers, F.A., van den Engh, G., Nolte-'t Hoen, E.N.M.and Wauben, M.H.M.
*Cytometry Part A,**89A**, 135-147 (2016)*

Submicron-sized vesicles released by cells are increasingly recognized for their role in intercellular communication and as biomarkers of disease. Methods for high-throughput, multi-parameter analysis of such extracellular vesicles (EVs) are crucial to further investigate their diversity and function. We recently developed a high-resolution flow cytometry-based method (using a modified BD Influx) for quantitative and qualitative analysis of EVs. The fact that the majority of EVs is <200 nm in size requires special attention with relation to specific conditions of the flow cytometer, as well as sample concentration and event rate. In this study, we investigated how (too) high particle concentrations affect high-resolution flow cytometry-based particle quantification and characterization. Increasing concentrations of submicron-sized particles (beads, liposomes, and EVs) were measured to identify coincidence and swarm effects, caused by the concurrent presence of multiple particles in the measuring spot. As a result, we demonstrate that analysis of highly concentrated samples resulted in an underestimation of the number of particles and an interdependent overestimation of light scattering and fluorescence signals. On the basis of this knowledge, and by varying nozzle size and sheath pressure, we developed a strategy for high-resolution flow cytometric sorting of submicron-sized particles. Using the adapted sort settings, subsets of EVs differentially labeled with two fluorescent antibodies could be sorted to high purity. Moreover, sufficient numbers of EVs could be sorted for subsequent analysis by western blotting. In conclusion, swarm effects that occur when measuring high particle concentrations severely hamper EV quantification and characterization. These effects can be easily overlooked without including proper controls (e.g., sample dilution series) or tools (e.g., oscilloscope). Providing that the event rate is well controlled, the sorting strategy we propose here indicates that high-resolution flow cytometric sorting of different EV subsets is feasible.

3.2463 Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons
Krishnaswami, S.R. et al
*Nature Protocols, **11(3)**, 499-524 (2016)*

A protocol is described for sequencing the transcriptome of a cell nucleus. Nuclei are isolated from specimens and sorted by FACS, cDNA libraries are constructed and RNA-seq is performed, followed by data analysis. Some steps follow published methods (Smart-seq2 for cDNA synthesis and Nextera XT barcoded library preparation) and are not described in detail here. Previous single-cell approaches for RNA-seq from tissues include cell dissociation using protease treatment at 30 °C, which is known to alter

the transcriptome. We isolate nuclei at 4 °C from tissue homogenates, which cause minimal damage. Nuclear transcriptomes can be obtained from postmortem human brain tissue stored at −80 °C, making brain archives accessible for RNA-seq from individual neurons. The method also allows investigation of biological features unique to nuclei, such as enrichment of certain transcripts and precursors of some noncoding RNAs. By following this procedure, it takes about 4 d to construct cDNA libraries that are ready for sequencing.

3.2464 Myo19 is an outer mitochondrial membrane motor and effector of starvation-induced filopodia
Shneyer, B.I., Usaj, M. and henn, A.
*J. Cell Sci., **129**, 543-556 (2016)*

Mitochondria respond to environmental cues and stress conditions. Additionally, the disruption of the mitochondrial network dynamics and its distribution is implicated in a variety of neurodegenerative diseases. Here, we reveal a new function for Myo19 in mitochondrial dynamics and localization during the cellular response to glucose starvation. Ectopically expressed Myo19 localized with mitochondria to the tips of starvation-induced filopodia. Corollary to this, RNA interference (RNAi)-mediated knockdown of Myo19 diminished filopodia formation without evident effects on the mitochondrial network. We analyzed the Myo19–mitochondria interaction, and demonstrated that Myo19 is uniquely anchored to the outer mitochondrial membrane (OMM) through a 30–45-residue motif, indicating that Myo19 is a stably attached OMM molecular motor. Our work reveals a new function for Myo19 in mitochondrial positioning under stress.

3.2465 CD44-mediated monocyte transmigration across Cryptococcus neoformans-infected brain microvascular endothelial cells is enhanced by HIV-1 gp41-I90 ectodomain
He, X., Shi, X., Puthiyakunnon, S., Zhang, L., Zeng, Q., Li, Y., Boddu, S., Qiu, J., Lai, Z., Ma, C., Xie, Y., Long, M., Du, M., Huang, S-H. and Cao, H.
*J. Biomed. Sci., **23**:28 (2016)*

Background
Cryptococcus neoformans (Cn) is an important opportunistic pathogen in the immunocompromised people, including AIDS patients, which leads to fatal cryptococcal meningitis with high mortality rate. Previous researches have shown that HIV-1 gp41-I90 ectodomain can enhance Cn adhesion to and invasion of brain microvascular endothelial cell (BMEC), which constitutes the blood brain barrier (BBB). However, little is known about the role of HIV-1 gp41-I90 in the monocyte transmigration across Cn-infected BBB. In the present study, we provide evidence that HIV-1 gp41-I90 and Cn synergistically enhance monocytes transmigration across the BBB in vitro and in vivo. The underlying mechanisms for this phenomenon require further study.
Methods
In this study, the enhancing role of HIV-1 gp41-I90 in monocyte transmigration across Cn-infected BBB was demonstrated by performed transmigration assays in vitro and in vivo.
Results
Our results showed that the transmigration rate of monocytes are positively associated with Cn and/or HIV-1 gp41-I90, the co-exposure (HIV-1 gp41-I90 + Cn) group showed a higher THP-1 transmigration rate ($P < 0.01$). Using CD44 knock-down HBMEC or CD44 inhibitor Bikunin in the assay, the facilitation of transmigration rates of monocyte enhanced by HIV-1 gp41-I90 was significantly suppressed. Western blotting analysis and biotin/avidin enzyme-linked immunosorbent assays (BA-ELISAs) showed that Cn and HIV-1 gp41-I90 could increase the expression of CD44 and ICAM-1 on the HBMEC. Moreover, Cn and/or HIV-1 gp41-I90 could also induce CD44 redistribution to the membrane lipid rafts. By establishing the mouse cryptococcal meningitis model, we found that HIV-1 gp41-I90 and Cn could synergistically enhance the monocytes transmigration, increase the BBB permeability and injury in vivo.
Conclusions
Collectively, our findings suggested that HIV-1 gp41-I90 ectodomain can enhance the transmigration of THP-1 through Cn-infected BBB, which may be mediated by CD44. This novel study enlightens the future prospects to elaborate the inflammatory responses induced by HIV-1 gp41-I90 ectodomain and to effectively eliminate the opportunistic infections in AIDS patients.

3.2466 Characterization and Vaccine Potential of Outer Membrane Vesicles Produced by Haemophilus parasuis
McCaig, W.D., Loving, C.L., Hughes, H.R. and Brockmeier, S.L.
*PloS One, **11(3)**, e0149132 (2016)*

Haemophilus parasuis is a Gram-negative bacterium that colonizes the upper respiratory tract of swine and is capable of causing a systemic infection, resulting in high morbidity and mortality. *H. parasuis* isolates display a wide range of virulence and virulence factors are largely unknown. Commercial bacterins are often used to vaccinate swine against *H. parasuis*, though strain variability and lack of cross-reactivity can make this an ineffective means of protection. Outer membrane vesicles (OMV) are spherical structures naturally released from the membrane of bacteria and OMV are often enriched in toxins, signaling molecules and other bacterial components. Examination of OMV structures has led to identification of virulence factors in a number of bacteria and they have been successfully used as subunit vaccines. We have isolated OMV from both virulent and avirulent strains of *H. parasuis*, have examined their protein content and assessed their ability to induce an immune response in the host. Vaccination with purified OMV derived from the virulent *H. parasuis* Nagasaki strain provided protection against challenge with a lethal dose of the bacteria.

3.2467 Gestational Diabetes Mellitus Is Associated With Changes in the Concentration and Bioactivity of Placenta-Derived Exosomes in Maternal Circulation Across Gestation
Salomon, C., Scholz-Romero, K., Sarker, S., Sweeney, E., Kobayashi, M., Correa, P., Longo, S., Duncombe, G., Mitchell, M.D., Rice, G.E. and Illanes, S.E.
*Diabetes, **65**, 598-609 (2016)*

Although there is significant interest in elucidating the role of placenta-derived exosomes (PdEs) during pregnancy, the exosomal profile in pregnancies complicated by gestational diabetes mellitus (GDM) remains to be established. The aim of this study was to compare the gestational-age profile of PdEs in maternal plasma of GDM with normal pregnancies and to determine the effect of exosomes on cytokine release from human umbilical vein endothelial cells. A prospective cohort of patients was sampled at three time points during pregnancy for each patient (i.e., 11–14, 22–24, and 32–36 weeks' gestation). A retrospective stratified study design was used to quantify exosomes present in maternal plasma of normal (n = 13) and GDM (n = 7) pregnancies. Gestational age and pregnancy status were identified as significant factors contributing to variation in plasma exosome concentration (ANOVA, $P < 0.05$). Post hoc analyses established that PdE concentration increased during gestation in both normal and GDM pregnancies; however, the increase was significantly greater in GDM (∼2.2-fold, ∼1.5-fold, and ∼1.8-fold greater at each gestational age compared with normal pregnancies). Exosomes isolated from GDM pregnancies significantly increased the release of proinflammatory cytokines from endothelial cells. Although the role of exosomes during GDM remains to be fully elucidated, exosome profiles may be of diagnostic utility for screening asymptomatic populations.

3.2468 Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes
Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J.P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M. and Thery, C.
*PNAS, **113**, E968-E977 (2016)*

Extracellular vesicles (EVs) have become the focus of rising interest because of their numerous functions in physiology and pathology. Cells release heterogeneous vesicles of different sizes and intracellular origins, including small EVs formed inside endosomal compartments (i.e., exosomes) and EVs of various sizes budding from the plasma membrane. Specific markers for the analysis and isolation of different EV populations are missing, imposing important limitations to understanding EV functions. Here, EVs from human dendritic cells were first separated by their sedimentation speed, and then either by their behavior upon upward floatation into iodixanol gradients or by immuno-isolation. Extensive quantitative proteomic analysis allowing comparison of the isolated populations showed that several classically used exosome markers, like major histocompatibility complex, flotillin, and heat-shock 70-kDa proteins, are similarly present in all EVs. We identified proteins specifically enriched in small EVs, and define a set of five protein categories displaying different relative abundance in distinct EV populations. We demonstrate the presence of exosomal and nonexosomal subpopulations within small EVs, and propose their differential separation by immuno-isolation using either CD63, CD81, or CD9. Our work thus provides guidelines to define subtypes of EVs for future functional studies.

3.2469 Positive-strand RNA viruses stimulate host phosphatidylcholine synthesis at viral replication sites
Zhang, J., Zhang, Z., Chukkapalli, V., Nchoutmboube, J.A., Li, J., Randall, G., Belov, G.A. and Wang, X.
*PNAS, **113(8)**, E1064-E1073 (2016)*

All positive-strand RNA viruses reorganize host intracellular membranes to assemble their viral replication complexes (VRCs); however, how these viruses modulate host lipid metabolism to accommodate such membrane proliferation and rearrangements is not well defined. We show that a significantly increased phosphatidylcholine (PC) content is associated with brome mosaic virus (BMV) replication in both natural host barley and alternate host yeast based on a lipidomic analysis. Enhanced PC levels are primarily associated with the perinuclear ER membrane, where BMV replication takes place. More specifically, BMV replication protein 1a interacts with and recruits Cho2p (choline requiring 2), a host enzyme involved in PC synthesis, to the site of viral replication. These results suggest that PC synthesized at the site of VRC assembly, not the transport of existing PC, is responsible for the enhanced accumulation. Blocking PC synthesis by deleting the *CHO2* gene resulted in VRCs with wider diameters than those in wild-type cells; however, BMV replication was significantly inhibited, highlighting the critical role of PC in VRC formation and viral replication. We further show that enhanced PC levels also accumulate at the replication sites of hepatitis C virus and poliovirus, revealing a conserved feature among a group of positive-strand RNA viruses. Our work also highlights a potential broad-spectrum antiviral strategy that would disrupt PC synthesis at the sites of viral replication but would not alter cellular processes.

3.2470 Shp2 Associates with and Enhances Nephrin Tyrosine Phosphorylation and Is Necessary for Foot Process Spreading in Mouse Models of Podocyte Injury
Verma, R., Venkatareddy, M., Kalinowski, A., Patel, S.R., Salant, D.J. and Garg, P.
Mol. Cell. Biol., ***36(4)***, *596-614 (2016)*

In most forms of glomerular diseases, loss of size selectivity by the kidney filtration barrier is associated with changes in the morphology of podocytes. The kidney filtration barrier is comprised of the endothelial lining, the glomerular basement membrane, and the podocyte intercellular junction, or slit diaphragm. The cell adhesion proteins nephrin and neph1 localize to the slit diaphragm and transduce signals in a Src family kinase Fyn-mediated tyrosine phosphorylation-dependent manner. Studies in cell culture suggest nephrin phosphorylation-dependent signaling events are primarily involved in regulation of actin dynamics and lamellipodium formation. Nephrin phosphorylation is a proximal event that occurs both during development and following podocyte injury. We hypothesized that abrogation of nephrin phosphorylation following injury would prevent nephrin-dependent actin remodeling and foot process morphological changes. Utilizing a biased screening approach, we found nonreceptor Src homology 2 (sh2) domain-containing phosphatase Shp2 to be associated with phosphorylated nephrin. We observed an increase in nephrin tyrosine phosphorylation in the presence of Shp2 in cell culture studies. In the human glomerulopathies minimal-change nephrosis and membranous nephropathy, there is an increase in Shp2 phosphorylation, a marker of increased Shp2 activity. Mouse podocytes lacking Shp2 do not develop foot process spreading when subjected to podocyte injury *in vivo* using protamine sulfate or nephrotoxic serum (NTS). In the NTS model, we observed a lack of foot process spreading in mouse podocytes with Shp2 deleted and smaller amounts of proteinuria. Taken together, these results suggest that Shp2-dependent signaling events are necessary for changes in foot process structure and function following injury.

3.2471 A Role of TMEM16E Carrying a Scrambling Domain in Sperm Motility
Gyobu, S., Miyata, H., Ikawa, M., Yamazaki, D., Takeshima, H., Suzuki, J. and nagata, S.
Mol. Cell. Biol., ***36(4)***, *645-659 (2016)*

Transmembrane protein 16E (TMEM16E) belongs to the TMEM16 family of proteins that have 10 transmembrane regions and appears to localize intracellularly. Although TMEM16E mutations cause bone fragility and muscular dystrophy in humans, its biochemical function is unknown. In the TMEM16 family, TMEM16A and -16B serve as Ca^{2+}-dependent Cl^- channels, while TMEM16C, -16D, -16F, -16G, and -16J support Ca^{2+}-dependent phospholipid scrambling. Here, we show that TMEM16E carries a segment composed of 35 amino acids homologous to the scrambling domain in TMEM16F. When the corresponding segment of TMEM16A was replaced by this 35-amino-acid segment of TMEM16E, the chimeric molecule localized to the plasma membrane and supported Ca^{2+}-dependent scrambling. We next established *TMEM16E*-deficient mice, which appeared to have normal skeletal muscle. However, fertility was decreased in the males. We found that TMEM16E was expressed in germ cells in early spermatogenesis and thereafter and localized to sperm tail. *TMEM16E*$^{-/-}$ sperm showed no apparent defect in morphology, beating, mitochondrial function, capacitation, or binding to zona pellucida. However, they showed reduced motility and inefficient fertilization of cumulus-free but zona-intact eggs *in vitro*. Our

results suggest that TMEM16E may function as a phospholipid scramblase at inner membranes and that its defect affects sperm motility.

3.2472 Isolation and Analysis of Detergent-Resistant Membrane Fractions
Aureli, M., Grassi, S., Sonnino, S. and Prinetti, A.
Methods in Mol. Biol., ***1376****, 107-131 (2016)*

The hypothesis that the Golgi apparatus is capable of sorting proteins and sending them to the plasma membrane through "lipid rafts," membrane lipid domains highly enriched in glycosphingolipids, sphingomyelin, ceramide, and cholesterol, was formulated by van Meer and Simons in 1988 and came to a turning point when it was suggested that lipid rafts could be isolated thanks to their resistance to solubilization by some detergents, namely Triton X-100. An incredible number of papers have described the composition and properties of detergent-resistant membrane fractions. However, the use of this method has also raised the fiercest criticisms. In this chapter, we would like to discuss the most relevant methodological aspects related to the preparation of detergent-resistant membrane fractions, and to discuss the importance of discriminating between what is present on a cell membrane and what we can prepare from cell membranes in a laboratory tube.

3.2473 Extensible Multiplex Real-time PCR of MicroRNA Using Microparticles
Jung, S., Kim, J., Lee, D.J., Oh, E.H., Lim, H., Kim, K.P., Choi, N., Kim, T.S. and Kim, S.K.
Scientific Reports, ***6****;22975 (2016)*

Multiplex quantitative real-time PCR (qPCR), which measures multiple DNAs in a given sample, has received significant attention as a mean of verifying the rapidly increasing genetic targets of interest in single phenotype. Here we suggest a readily extensible qPCR for the expression analysis of multiple microRNA (miRNA) targets using microparticles of primer-immobilized networks as discrete reactors. Individual particles, 200~500 μm in diameter, are identified by two-dimensional codes engraved into the particles and the non-fluorescent encoding allows high-fidelity acquisition of signal in real-time PCR. During the course of PCR, the amplicons accumulate in the volume of the particles with high reliability and amplification efficiency over 95%. In a quick assay comprising of tens of particles holding different primers, each particle brings the independent real-time amplification curve representing the quantitative information of each target. Limited amount of sample was analyzed simultaneously in single chamber through this highly multiplexed qPCR; 10 kinds of miRNAs from purified extracellular vesicles (EVs).

3.2474 Up-regulation of Hnf1α gene expression in the liver of rats with experimentally induced chronic renal failure – A possible link between circulating PCSK9 and triacylglycerol concentrations
Sucajtys-Szulc, E., Szolkiewicz, M. and Swierczynski, J.
Atherosclerosis, ***248****, 17-26 (2016)*

Background
The aim of this study was to verify if an increase in *Hnf1α* gene expression could be a possible link between circulating proprotein convertase subtilisin/kexin type 9 (PCSK9) and TAGs concentrations in chronic renal failure (CRF).
Methods
Rats underwent 5/6 nephrectomy or a sham surgery. Liver expressions of *Pcsk9, Mttp, ApoB-100, Hnf1α, Hnf4α,* lipogenic enzymes and *β-actin* genes were quantified by qPCR. Liver levels of proteins coding by these genes were analyzed by Western blotting. Serum apoB-100 and PCSK9 concentration were estimated with an immunoassay.
Results
CRF rats showed an increase in circulating concentrations of TAGs, VLDL, apoB-100 and PCSK9, along with an enhanced liver VLDL-TAG secretion rate and a coordinated liver up-regulation of genes coding: a) lipogenic enzymes; b) *Mttp* and *ApoB-100*; c) *Pcsk9*; d) *Hnf1α* and *Hnf4α*. Positive correlations were found between serum creatinine concentrations and: a) the liver levels of HNF1*α* mRNA ($r = 0.79$, $p < 0.01$) and HNF4*α* ($r = 0.76$, $p < 0.01$); b) the liver levels of PCSK9 mRNA ($r = 0.88$, $p < 0.01$) and serum PCSK9 concentrations ($r = 0.73$, $p < 0.01$); c) the liver levels of apoB-100 mRNA ($r = 0.83$, $p < 0.01$) and serum apoB-100 concentrations ($r = 0.87$, $p < 0.01$). Clofibrate treatment was shown to concomitantly decrease the liver levels of HNF1α, HNF4α and PCSK9 mRNA, as well as serum PCSK9, TAGs and total cholesterol concentrations in CRF rats.
Conclusion

The results presented are consistent with a cause-effect relationship between the enhanced liver expression of *Hnf1α* gene and its target genes the products of which are involved in synthesis, assembly and secretion of VLDL, as well as Pcsk9 gene in CRF rats. This may at least in part explain an association between circulating PCSK9 and TAGs in CRF rats and possibly also in humans with chronic kidney disease (CKD).

3.2475 Annexin A2 Limits Neutrophil Transendothelial Migration by Organizing the Spatial Distribution of ICAM-1

Heemskerk, N., Asimuddin, A., Oort, c., van Rijssel, J. and van Buul, J.D.
J. Immunol., ***196(6)***, *2767-2778 (2016)*

ICAM-1 is required for firm adhesion of leukocytes to the endothelium. However, how the spatial organization of endothelial ICAM-1 regulates leukocyte adhesion is not well understood. In this study, we identified the calcium-effector protein annexin A2 as a novel binding partner for ICAM-1. ICAM-1 clustering promotes the ICAM-1–annexin A2 interaction and induces translocation of ICAM-1 into caveolin-1–rich membrane domains. Depletion of endothelial annexin A2 using RNA interference enhances ICAM-1 membrane mobility and prevents the translocation of ICAM-1 into caveolin-1–rich membrane domains. Surprisingly, this results in increased neutrophil adhesion and transendothelial migration under flow conditions and reduced crawling time, velocity, and lateral migration distance of neutrophils on the endothelium. In conclusion, our data show that annexin A2 limits neutrophil transendothelial migration by organizing the spatial distribution of ICAM-1.

3.2476 Disturbance of proteasomal and autophagic protein degradation pathways by amyotrophic lateral sclerosis-linked mutations in ubiquilin 2

Osaka, M., Ito, D. and Suzuki, N.
Biochem. Biophys. Res. Comm., ***1472***, *324-331 (2016)*

Ubiquilin (UBQLN), a member of the ubiquitin-like (UBL)-ubiquitin-associated (UBA) family, is a dual regulator of both the proteasomal and autophagic branches of the cellular protein degradation system. Mutations in the *UBQLN2* gene encoding ubiquilin 2 cause X-linked amyotrophic lateral sclerosis (ALS)/frontotemporal dementia (FTD), and UBQLN2-positive inclusions have been identified in ALS patients with UBQLN2 mutations as well as in cases of both familial and sporadic ALS without UBQLN2 mutations. Compelling evidence links UBQLN2 to disturbance of the protein quality control network in neurons, but the pathomechanisms remain obscure. This study aimed to clarify how ALS-linked mutations in UBQLN2 affect the protein degradation system. Overexpression of a UBQLN2 with ALS-associated mutations resulted in the accumulation of polyubiquitinated proteins in neuronal cells, including the ALS-associated protein TDP-43. This effect was dependent on the UBA domain but not on inclusion formation. Immunocytochemistry and protein fractionation analysis of IVm-UBQLN2 cellular distribution indicated that it sequesters ubiquitinated substrates from both the proteasomal and autophagic branches of the protein degradation system, resulting in accumulation of polyubiquitinated substrates. These findings provide a molecular basis for the development of ALS/FTD-associated proteinopathy and establish novel therapeutic targets for ALS.

3.2477 Enterovirus 71 induces dsRNA/PKR-dependent cytoplasmic redistribution of GRP78/BiP to promote viral replication

Jheng, J-R., Wang, S-C., Jheng, C-R. and Horng, J-T.
Emerging Microbes and Infections, ***5***, *e23 (2016)*

GRP78/BiP is an endoplasmic reticulum (ER) chaperone protein with the important function of maintaining ER homeostasis, and the overexpression of GRP78/BiP alleviates ER stress. Our previous studies showed that infection with enterovirus 71 (EV71), a (+)RNA picornavirus, induced GRP78/BiP upregulation; however, ectopic GRP78/BiP overexpression in ER downregulates virus replication and viral particle formation. The fact that a virus infection increases GRP78/BiP expression, which is unfavorable for virus replication, is counterintuitive. In this study, we found that the GRP78/BiP protein level was elevated in the cytoplasm instead of in the ER in EV71-infected cells. Cells transfected with polyinosinic–polycytidylic acid, a synthetic analog of replicative double-stranded RNA (dsRNA), but not with viral proteins, also exhibited upregulation and elevation of GRP78/BiP in the cytosol. Our results further demonstrate that EV71 infections induce the dsRNA/protein kinase R-dependent cytosolic accumulation of GRP78/BiP. The overexpression of a GRP78/BiP mutant lacking a KDEL retention signal failed to inhibit both dithiothreitol-induced eIF2α phosphorylation and viral replication in the context of viral protein

synthesis and viral titers. These data revealed that EV71 infection might cause upregulation and aberrant redistribution of GRP78/BiP to the cytosol, thereby facilitating virus replication.

3.2478 Residual matrix from different separation techniques impacts exosome biological activity
Paolini, L., Zendrini, A., Di Noto, G., Busatto, S., Lottini, E., Radeghieri, A., Dossi, A., Caneschi, A., Ricotta, D. and Bergese, P.
*Scientific Reports, **6**:23550 (2016)*

Exosomes are gaining a prominent role in research due to their intriguing biology and several therapeutic opportunities. However, their accurate purification from body fluids and detailed physicochemical characterization remain open issues. We isolated exosomes from serum of patients with Multiple Myeloma by four of the most popular purification methods and assessed the presence of residual contaminants in the preparations through an ad hoc combination of biochemical and biophysical techniques - including Western Blot, colloidal nanoplasmonics, atomic force microscopy (AFM) and scanning helium ion microscopy (HIM). The preparations obtained by iodixanol and sucrose gradients were highly pure. To the contrary, those achieved with limited processing (serial centrifugation or one step precipitation kit) resulted contaminated by a residual matrix, embedding the exosomes. The contaminated preparations showed lower ability to induce NfkB nuclear translocation in endothelial cells with respect to the pure ones, probably because the matrix prevents the interaction and fusion of the exosomes with the cell membrane. These findings suggest that exosome preparation purity must be carefully assessed since it may interfere with exosome biological activity. Contaminants can be reliably probed only by an integrated characterization approach aimed at both the molecular and the colloidal length scales.

3.2479 SGEF Is Regulated via TWEAK/Fn14/NF-κB Signaling and Promotes Survival by Modulation of the DNA Repair Response to Temozolomide
Ensign, S.P.F., Roos, A., Mathews, I.T., Dhruv, H.D., Tuncali, S., Sarkaria, J.N., Symons, M.H., Loftus, J.C., Berens, M.E. and Tran, N.L.
Mol. Cancer Res., 14(3), 302-312 (2016)

Glioblastoma (GB) is the highest grade and most common form of primary adult brain tumors. Despite surgical removal followed by concomitant radiation and chemotherapy with the alkylating agent temozolomide, GB tumors develop treatment resistance and ultimately recur. Impaired response to treatment occurs rapidly, conferring a median survival of just fifteen months. Thus, it is necessary to identify the genetic and signaling mechanisms that promote tumor resistance to develop targeted therapies to combat this refractory disease. Previous observations indicated that SGEF (ARHGEF26), a RhoG-specific guanine nucleotide exchange factor (GEF), is overexpressed in GB tumors and plays a role in promoting TWEAK-Fn14–mediated glioma invasion. Here, further investigation revealed an important role for SGEF in glioma cell survival. SGEF expression is upregulated by TWEAK-Fn14 signaling via NF-κB activity while shRNA-mediated reduction of SGEF expression sensitizes glioma cells to temozolomide-induced apoptosis and suppresses colony formation following temozolomide treatment. Nuclear SGEF is activated following temozolomide exposure and complexes with the DNA damage repair (DDR) protein BRCA1. Moreover, BRCA1 phosphorylation in response to temozolomide treatment is hindered by SGEF knockdown. The role of SGEF in promoting chemotherapeutic resistance highlights a heretofore unappreciated driver, and suggests its candidacy for development of novel targeted therapeutics for temozolomide-refractory, invasive GB cells.

3.2480 Vesicle Size Regulates Nanotube Formation in the Cell
Su, Q.P., Su, W., Ji, Q., Xue, B., Jiang, D., Zhu, Y., Lou, J., Yu, L. and Sun, Y.
*Scientific Reports, **6**:24002 (2016)*

Intracellular membrane nanotube formation and its dynamics play important roles for cargo transportation and organelle biogenesis. Regarding the regulation mechanisms, while much attention has been paid on the lipid composition and its associated protein molecules, effects of the vesicle size has not been studied in the cell. Giant unilamellar vesicles (GUVs) are often used for in vitro membrane deformation studies, but they are much larger than most intracellular vesicles and the in vitro studies also lack physiological relevance. Here, we use lysosomes and autolysosomes, whose sizes range between 100 nm and 1 μm, as model systems to study the size effects on nanotube formation both in vivo and in vitro. Single molecule observations indicate that driven by kinesin motors, small vesicles (100–200 nm) are mainly transported along the tracks while a remarkable portion of large vesicles (500–1000 nm) form nanotubes. This size effect is further confirmed by in vitro reconstitution assays on liposomes and purified lysosomes and

autolysosomes. We also apply Atomic Force Microscopy (AFM) to measure the initiation force for nanotube formation. These results suggest that the size-dependence may be one of the mechanisms for cells to regulate cellular processes involving membrane-deformation, such as the timing of tubulation-mediated vesicle recycling.

3.2481 Differential Responses of Pattern Recognition Receptors to Outer Membrane Vesicles of Three Periodontal Pathogens
Cecil, J.D., O'Brien-Simpson, N., Lenzo, J.C., Holden, J.A., Chen, Y-Y., Singleton, W., gause, K.T., Yan, Y., Caruso, F. and Reynolds, E.C.
*PloS One, **11(4)**, e0151967 (2016)*

Highly purified outer membrane vesicles (OMVs) of the periodontal pathogens, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* were produced using tangential flow ultrafiltration, ultracentrifugation and Optiprep density gradient separation. Cryo-TEM and light scattering showed OMVs to be single lipid-bilayers with modal diameters of 75 to 158 nm. Enumeration of OMVs by nanoparticle flow-cytometry at the same stage of late exponential culture indicated that *P. gingivalis* was the most prolific OMV producer. *P. gingivalis* OMVs induced strong TLR2 and TLR4-specific responses and moderate responses in TLR7, TLR8, TLR9, NOD1 and NOD2 expressing-HEK-Blue cells. Responses to *T. forsythia* OMVs were less than those of *P. gingivalis* and *T. denticola* OMVs induced only weak responses. Compositional analyses of OMVs from the three pathogens demonstrated differences in protein, fatty acids, lipopolysaccharide, peptidoglycan fragments and nucleic acids. Periodontal pathogen OMVs induced differential pattern recognition receptor responses that have implications for their role in chronic periodontitis.

3.2482 Sialic Acid Glycobiology Unveils Trypanosoma cruzi Trypomastigote Membrane Physiology
Lantos, A.B., Carlevaro, G., Araoz, B., Diaz, P.R., de los Milagros Camara, M., Buscaglia, C.A., Bossi, M., Yu, H., Chen, X., Bertozzi, C.R., Mucci, J. and Campetella, O.
*PloS Pathogens, **12(4)**, e1005550 (2016)*

Trypanosoma cruzi, the flagellate protozoan agent of Chagas disease or American trypanosomiasis, is unable to synthesize sialic acids *de novo*. Mucins and *trans*-sialidase (TS) are substrate and enzyme, respectively, of the glycobiological system that scavenges sialic acid from the host in a crucial interplay for *T. cruzi* life cycle. The acquisition of the sialyl residue allows the parasite to avoid lysis by serum factors and to interact with the host cell. A major drawback to studying the sialylation kinetics and turnover of the trypomastigote glycoconjugates is the difficulty to identify and follow the recently acquired sialyl residues. To tackle this issue, we followed an unnatural sugar approach as bioorthogonal chemical reporters, where the use of azidosialyl residues allowed identifying the acquired sugar. Advanced microscopy techniques, together with biochemical methods, were used to study the trypomastigote membrane from its glycobiological perspective. Main sialyl acceptors were identified as mucins by biochemical procedures and protein markers. Together with determining their shedding and turnover rates, we also report that several membrane proteins, including TS and its substrates, both glycosylphosphatidylinositol-anchored proteins, are separately distributed on parasite surface and contained in different and highly stable membrane microdomains. Notably, labeling for α(1,3)Galactosyl residues only partially colocalize with sialylated mucins, indicating that two species of glycosylated mucins do exist, which are segregated at the parasite surface. Moreover, sialylated mucins were included in lipid-raft-domains, whereas TS molecules are not. The location of the surface-anchored TS resulted too far off as to be capable to sialylate mucins, a role played by the shed TS instead. Phosphatidylinositol-phospholipase-C activity is actually not present in trypomastigotes. Therefore, shedding of TS occurs via microvesicles instead of as a fully soluble form.

3.2483 Extracellular vesicles during Herpes Simplex Virus type 1 infection: an inquire
Kalamvoki, M. and Deschamps, T.
*Virol. J., **13**:63 (2016)*

Extracellular vesicles are defined as a heterogeneous group of vesicles that are released by prokaryotic to higher eukaryotic cells and by plant cells in an evolutionary conserved manner. The significance of these vesicles lies in their capacity to transfer selected cargo composed of proteins, lipids and nucleic acids to both recipient and parent cells and to influence various physiological and pathological functions. Microorganisms such as parasites, fungi and protozoa and even single cell organisms such as bacteria generate extracellular vesicles. In addition, several viruses have evolved strategies to hijack the extracellular vesicles for egress or to alter the surrounding environment. The thesis of this article is that: a)

during HSV-1 infection vesicles are delivered from infected to uninfected cells that influence the infection; b) the cargo of these vesicles consists of viral and host transcripts (mRNAs, miRNAs and non-coding RNAs) and proteins including innate immune components, such as STING; and c) the viral vesicles carry the tetraspanins CD9, CD63 and CD81, which are considered as markers of exosomes. Therefore, we assume that the STING-carrying vesicles, produced during HSV-1 infection, are reminiscent to exosomes. The presumed functions of the exosomes released from HSV-1 infected cells include priming the recipient cells and accelerating antiviral responses to control the dissemination of the virus. This may be one strategy used by the virus to prevent the elimination by the host and establish persistent infection. In conclusion, the modification of the cargo of exosomes appears to be part of the strategy that HSV-1 has evolved to establish lifelong persistent infections into the human body to ensure successful dissemination between individuals.

3.2484 Identification of the novel activity-driven interaction between synaptotagmin 1 and presenilin 1 links calcium, synapse, and amyloid beta

Kuzuya, A., Zoltowska, K.M., Post, K.L., Arimon, M., Li, X., Svirsky, S., Maesako, M., Muzikansky, A., Gautam, V., Kovacs, D., Hyman, T. and Berezovska, O.
BMC Biology, ***14***:*25 (2016)*

Background
Synaptic loss strongly correlates with memory deterioration. Local accumulation of amyloid β (Aβ) peptide, and neurotoxic Aβ42 in particular, due to abnormal neuronal activity may underlie synaptic dysfunction, neurodegeneration, and memory impairments. To gain an insight into molecular events underlying neuronal activity-regulated Aβ production at the synapse, we explored functional outcomes of the newly discovered calcium-dependent interaction between Alzheimer's disease-associated presenilin 1 (PS1)/γ-secretase and synaptic vesicle proteins.
Results
Mass spectrometry screen of mouse brain lysates identified synaptotagmin 1 (Syt1) as a novel synapse-specific PS1-binding partner that shows Ca^{2+}-dependent PS1 binding profiles in vitro and in vivo. We found that Aβ level, and more critically, conformation of the PS1 and the $Aβ_{42/40}$ ratio, are affected by Syt1 overexpression or knockdown, indicating that Syt1 and its interaction with PS1 might regulate Aβ production at the synapse. Moreover, β-secretase 1 (BACE1) stability, β- and γ-secretase activity, as well as intracellular compartmentalization of PS1 and BACE1, but not of amyloid precursor protein (APP), nicastrin (Nct), presenilin enhancer 2 (Pen-2), or synaptophysin (Syp) were altered in the absence of Syt1, suggesting a selective effect of Syt1 on PS1 and BACE1 trafficking.
Conclusions
Our findings identify Syt1 as a novel Ca^{2+}-sensitive PS1 modulator that could regulate synaptic Aβ, opening avenues for novel and selective synapse targeting therapeutic strategies.

3.2485 Sng1 associates with Nce102 to regulate the yeast Pkh-Ypk signalling module in response to sphingolipid status

Garcia-marques, S., Randez-Gil, F., Dupont, S., Garre, E. and Prieto, J.A.
Biochim. Biophys. Acta, ***1863***, *1319-1333 (2016)*

All cells are delimited by biological membranes, which are consequently a primary target of stress-induced damage. Cold alters membrane functionality by decreasing lipids fluidity and the activity of membrane proteins. In *Saccharomyces cerevisiae*, evidence links sphingolipid homeostasis and membrane phospholipid asymmetry to the activity of the Ypk1/2 proteins, the yeast orthologous of the mammalian SGK1-3 kinases. Their regulation is mediated by different protein kinases, including the PDK1 orthologous Pkh1/2p, and requires the function of protein effectors, among them Nce102p, a component of the sphingolipid sensor machinery. Nevertheless, the mechanisms and the actors involved in Pkh/Ypk regulation remain poorly defined. Here, we demonstrate that Sng1, a transmembrane protein, is an effector of the Pkh/Ypk module and identify the phospholipids asymmetry as key for yeast cold adaptation. Overexpression of *SNG1* impairs phospholipid flipping, reduces reactive oxygen species (ROS) and improves, in a Pkh-dependent manner, yeast growth in myriocin-treated cells, suggesting that excess Sng1p stimulates the Pkh/Ypk signalling. Furthermore, we link these effects to the association of Sng1p with Nce102p. Indeed, we found that Sng1p interacts with Nce102p both physically and genetically. Moreover, mutant *nce102* Δ *sng1* Δ cells show features of impaired Pkh/Ypk signalling, including increased ROS accumulation, reduced life span and defects in Pkh/Ypk-controlled regulatory pathways. Finally, myriocin-induced hyperphosphorylation of Ypk1 and Orm2, which controls sphingolipid homeostasis, does not

occur in *nce102* Δ *sng1* Δ cells. Hence, both Nce102p and Sng1p participate in a regulatory circuit that controls the activity of the Pkh/Ypk module and their function is required in response to sphingolipid status.

3.2486 Inflammatory properties of inhibitor of DNA binding 1 secreted by synovial fibroblasts in rheumatoid arthritis

Edhayan, G., Ohara, R.A., Stinson, W.A., Amin, M.A., Isozaki, T., Ha, C.M., Haines III, G.K., Morgan, R., Campbell, P.L., Arbab, A.S., Friday, S.C., Fox, D.A. and Ruth, J.H.
*Arthritis Res. & Therapy, **18**:87 (2016)*

Background
Inhibitor of DNA binding 1 (Id1) is a nuclear protein containing a basic helix-loop-helix (bHLH) domain that regulates cell growth by selective binding and prevention of gene transcription. Sources of Id1 production in rheumatoid arthritis synovial tissue (RA ST) and its range of functional effects in RA remain to be clarified.
Methods
We analyzed Id1 produced from synovial fibroblasts and endothelial cells (ECs) with histology and real-time polymerase chain reaction (RT-PCR). Fibroblast supernatants subjected to differential centrifugation to isolate and purify exosomes were measured for Id1 by enzyme-linked immunosorbent assay (ELISA). Western blotting of Id1-stimulated ECs was performed to determine the kinetics of intracellular protein phosphorylation. EC intracellular signaling pathways induced by Id1 were subsequently targeted with silencing RNA (siRNA) for angiogenesis inhibition.
Results
By PCR and histologic analysis, we found that the primary source of Id1 in STs is from activated fibroblasts that correlate with inflammatory scores in human RA ST and in joints from K/BxN serum-induced mice. Normal (NL) and RA synovial fibroblasts increase Id1 production with stimulation by transforming growth factor beta (TGF-β). Most of the Id1 released by RA synovial fibroblasts is contained within exosomes. Endothelial progenitor cells (EPCs) and human dermal microvascular ECs (HMVECs) activate the Jnk signaling pathway in response to Id1, and Jnk siRNA reverses Id1-induced HMVEC vessel formation in Matrigel plugs in vivo.
Conclusions
Id1 is a pleotropic molecule affecting angiogenesis, vasculogenesis, and fibrosis. Our data shows that Id1 is not only an important nuclear protein, but also can be released from fibroblasts via exosomes. The ability of extracellular Id1 to activate signaling pathways expands the role of Id1 in the orchestration of tissue inflammation.

3.2487 Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms

Turnbull, L. et al
*Nature Communications, **7**:11220 (2016)*

Many bacteria produce extracellular and surface-associated components such as membrane vesicles (MVs), extracellular DNA and moonlighting cytosolic proteins for which the biogenesis and export pathways are not fully understood. Here we show that the explosive cell lysis of a sub-population of cells accounts for the liberation of cytosolic content in *Pseudomonas aeruginosa* biofilms. Super-resolution microscopy reveals that explosive cell lysis also produces shattered membrane fragments that rapidly form MVs. A prophage endolysin encoded within the R- and F-pyocin gene cluster is essential for explosive cell lysis. Endolysin-deficient mutants are defective in MV production and biofilm development, consistent with a crucial role in the biogenesis of MVs and liberation of extracellular DNA and other biofilm matrix components. Our findings reveal that explosive cell lysis, mediated through the activity of a cryptic prophage endolysin, acts as a mechanism for the production of bacterial MVs.

3.2488 Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection

Sodar, B.W. et al
*Scientific Reports, **6**:24316 (2016)*

Circulating extracellular vesicles have emerged as potential new biomarkers in a wide variety of diseases. Despite the increasing interest, their isolation and purification from body fluids remains challenging. Here we studied human pre-prandial and 4 hours postprandial platelet-free blood plasma samples as well as human platelet concentrates. Using flow cytometry, we found that the majority of circulating particles within the size range of extracellular vesicles lacked common vesicular markers. We identified most of

these particles as lipoproteins (predominantly low-density lipoprotein, LDL) which mimicked the characteristics of extracellular vesicles and also co-purified with them. Based on biophysical properties of LDL this finding was highly unexpected. Current state-of-the-art extracellular vesicle isolation and purification methods did not result in lipoprotein-free vesicle preparations from blood plasma or from platelet concentrates. Furthermore, transmission electron microscopy showed an association of LDL with isolated vesicles upon in vitro mixing. This is the first study to show co-purification and in vitro association of LDL with extracellular vesicles and its interference with vesicle analysis. Our data point to the importance of careful study design and data interpretation in studies using blood-derived extracellular vesicles with special focus on potentially co-purified LDL.

3.2489 Impairment of extramitochondrial oxidative phosphorylation in mouse rod outer segments by blue light irradiation

Calzia, D., Panfoli, I., heinig, N., Schumann, U., Ader, M., Traverso, C.E., Funk, R.H.W. and roehlecke, C.

*Biochimie, **125**, 171-178 (2016)*

Exposure to short wavelength light causes increased reactive oxygen intermediates production in the outer retina, particularly in the rod Outer Segments (OS). Consistently, the OS were shown to conduct aerobic ATP production through the ectopic expression of the electron transfer chain complexes I–IV and F_1F_o-ATP synthase. These facts prompted us to verify if the oxidative phosphorylation in the OS is implied in the oxidative damage of the blue-light (BL) treated OS, in an organotypic model of mouse retina.
Whole mouse eyeball cultures were treated with short wavelength BL (peak at 405 nm, output power 1 mW/cm^2) for 6 h. Immunogold transmission electron microscopy confirmed the expression of Complex I and F_1F_o-ATP synthase in the OS. *In situ* histochemical assays on unfixed sections showed impairment of respiratory Complexes I and II after BL exposure, both in the OS and IS, utilized as a control. Basal O_2 consumption and ATP synthesis were impaired in the OS purified from blue-light irradiated eyeball cultures. Electron transfer capacity between Complex I and II as well as activity of Complexes I and II was decreased in blue-light irradiated purified OS.
The severe malfunctioning of the OS aerobic respiratory capacity after 6 h BL treatment may be the consequence of a self-induced damage. BL exposure would cause an initial over-functioning of both the phototransduction and respiratory chain, with reactive oxygen species production. In a self-renewal vicious cycle, membrane and protein oxidative damage, proton leakage and uncoupling, would impair redox chains, perpetuating the damage and causing hypo-metabolism with eventual apoptosis of the rod. Data may shed new light on the rod-driven retinopathies such as Age Related Macular Degeneration, of which blue-light irradiated retina represents a model.

3.2490 Identification of CiaR Regulated Genes That Promote Group B Streptococcal Virulence and Interaction with Brain Endothelial Cells

Mu, R., Cutting, A.S., Del Rosario, Y., Villarino, N., Stewart, L., Weston, T.A., Patras, K.A. and Doran, K.S.

*PloS One, **11(4)**, e0153891 (2016)*

Group B *Streptococcus* (GBS) is a major causative agent of neonatal meningitis due to its ability to efficiently cross the blood-brain barrier (BBB) and enter the central nervous system (CNS). It has been demonstrated that GBS can invade human brain microvascular endothelial cells (hBMEC), a primary component of the BBB; however, the mechanism of intracellular survival and trafficking is unclear. We previously identified a two component regulatory system, CiaR/H, which promotes GBS intracellular survival in hBMEC. Here we show that a GBS strain deficient in the response regulator, CiaR, localized more frequently with Rab5, Rab7 and LAMP1 positive vesicles. Further, lysosomes isolated from hBMEC contained fewer viable bacteria following initial infection with the Δ*ciaR* mutant compared to the WT strain. To characterize the contribution of CiaR-regulated genes, we constructed isogenic mutant strains lacking the two most down-regulated genes in the CiaR-deficient mutant, SAN_2180 and SAN_0039. These genes contributed to bacterial uptake and intracellular survival. Furthermore, competition experiments in mice showed that WT GBS had a significant survival advantage over the Δ*2180* and Δ*0039* mutants in the bloodstream and brain.

3.2491 Sphingosine-1-Phosphate Lyase Deficient Cells as a Tool to Study Protein Lipid Interactions

Geri, M.J., Bittl, V., Kirchner, S., Sachenheimer, T., Brunner, H.L., Lüchtenborg, C., Özbalci, C., Wiedemann, H., Wegehingel, S., Nickel, w., haberkant, P., Schultz, C., Krüger, M. and Brügger, B.

*PloS One, **11(4)**, e0153009 (2016)*

Cell membranes contain hundreds to thousands of individual lipid species that are of structural importance but also specifically interact with proteins. Due to their highly controlled synthesis and role in signaling events sphingolipids are an intensely studied class of lipids. In order to investigate their metabolism and to study proteins interacting with sphingolipids, metabolic labeling based on photoactivatable sphingoid bases is the most straightforward approach. In order to monitor protein-lipid-crosslink products, sphingosine derivatives containing a reporter moiety, such as a radiolabel or a clickable group, are used. In normal cells, degradation of sphingoid bases via action of the checkpoint enzyme sphingosine-1-phosphate lyase occurs at position C2-C3 of the sphingoid base and channels the resulting hexadecenal into the glycerolipid biosynthesis pathway. In case the functionalized sphingosine looses the reporter moiety during its degradation, specificity towards sphingolipid labeling is maintained. In case degradation of a sphingosine derivative does not remove either the photoactivatable or reporter group from the resulting hexadecenal, specificity towards sphingolipid labeling can be achieved by blocking sphingosine-1-phosphate lyase activity and thus preventing sphingosine derivatives to be channeled into the sphingolipid-to-glycerolipid metabolic pathway. Here we report an approach using clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated nuclease Cas9 to create a sphingosine-1-phosphate lyase (*SGPL1*) HeLa knockout cell line to disrupt the sphingolipid-to-glycerolipid metabolic pathway. We found that the lipid and protein compositions as well as sphingolipid metabolism of *SGPL1* knock-out HeLa cells only show little adaptations, which validates these cells as model systems to study transient protein-sphingolipid interactions.

3.2492 **Phosphorylation of Ago2 and Subsequent Inactivation of let-7a RNP-Specific MicroRNAs Control Differentiation of Mammalian Sympathetic Neurons**
Patranabis, S. and Bhattacharyya, S.N.
Mol. Cell. Biol., ***36(8)****, 1260-1271 (2016)*

MicroRNAs (miRNAs) are small regulatory RNAs that regulate gene expression posttranscriptionally by base pairing to the target mRNAs in animal cells. *KRas*, an oncogene known to be repressed by let-7a miRNAs, is expressed and needed for the differentiation of mammalian sympathetic neurons and PC12 cells. We documented a loss of let-7a activity during this differentiation process without any significant change in the cellular level of let-7a miRNA. However, the level of Ago2, an essential component that is associated with miRNAs to form RNP-specific miRNA (miRNP) complexes, shows an increase with neuronal differentiation. In this study, differentiation-induced phosphorylation and the subsequent loss of miRNA from Ago2 were noted, and these accounted for the loss of miRNA activity in differentiating neurons. Neuronal differentiation induces the phosphorylation of mitogen-activated protein kinase p38 and the downstream kinase mitogen- and stress-activated protein kinase 1 (MSK1). This in turn upregulates the phosphorylation of Ago2 and ensures the dissociation of miRNA from Ago2 in neuronal cells. MSK1-mediated miRNP inactivation is a prerequisite for the differentiation of neuronal cells, where let-7a miRNA gets unloaded from Ago2 to ensure the upregulation of *KRas*, a target of let-7a. We noted that the inactivation of let-7a is both necessary and sufficient for the differentiation of sympathetic neurons.

3.2493 **P53- and mevalonate pathway–driven malignancies require Arf6 for metastasis and drug resistance**
Hashimoto, A., Oikawa, T., Hashimoto, S., Sugino, H., Yoshikawa, A., Otsuka, Y., Handa, H., Onodera, Y., Nam, J-M., Oneyama, C., Okada, M., Fukuda, M. and Sabe, H.
J. Cell Biol., ***213(1)****, 81-95 (2016)*

Drug resistance, metastasis, and a mesenchymal transcriptional program are central features of aggressive breast tumors. The GTPase Arf6, often overexpressed in tumors, is critical to promote epithelial–mesenchymal transition and invasiveness. The metabolic mevalonate pathway (MVP) is associated with tumor invasiveness and known to prenylate proteins, but which prenylated proteins are critical for MVP-driven cancers is unknown. We show here that MVP requires the Arf6-dependent mesenchymal program. The MVP enzyme geranylgeranyl transferase II (GGT-II) and its substrate Rab11b are critical for Arf6 trafficking to the plasma membrane, where it is activated by receptor tyrosine kinases. Consistently, mutant p53, which is known to support tumorigenesis via MVP, promotes Arf6 activation via GGT-II and Rab11b. Inhibition of MVP and GGT-II blocked invasion and metastasis and reduced cancer cell resistance against chemotherapy agents, but only in cells overexpressing Arf6 and components of the mesenchymal program. Overexpression of Arf6 and mesenchymal proteins as well as enhanced MVP activity correlated with poor patient survival. These results provide insights into the molecular basis of MVP-driven malignancy.

3.2494 **A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance**
Jang, C. et al
Nature Med., ***22(4)***, *421-426 (2016)*

Epidemiological and experimental data implicate branched-chain amino acids (BCAAs) in the development of insulin resistance, but the mechanisms that underlie this link remain unclear[1, 2, 3]. Insulin resistance in skeletal muscle stems from the excess accumulation of lipid species[4], a process that requires blood-borne lipids to initially traverse the blood vessel wall. How this trans-endothelial transport occurs and how it is regulated are not well understood. Here we leveraged PPARGC1a (also known as PGC-1α; encoded by *Ppargc1a*), a transcriptional coactivator that regulates broad programs of fatty acid consumption, to identify 3-hydroxyisobutyrate (3-HIB), a catabolic intermediate of the BCAA valine, as a new paracrine regulator of trans-endothelial fatty acid transport. We found that 3-HIB is secreted from muscle cells, activates endothelial fatty acid transport, stimulates muscle fatty acid uptake *in vivo* and promotes lipid accumulation in muscle, leading to insulin resistance in mice. Conversely, inhibiting the synthesis of 3-HIB in muscle cells blocks the ability of PGC-1α to promote endothelial fatty acid uptake. 3-HIB levels are elevated in muscle from *db/db* mice with diabetes and from human subjects with diabetes, as compared to those without diabetes. These data unveil a mechanism in which the metabolite 3-HIB, by regulating the trans-endothelial flux of fatty acids, links the regulation of fatty acid flux to BCAA catabolism, providing a mechanistic explanation for how increased BCAA catabolic flux can cause diabetes.

3.2495 **Ca2+-regulated lysosome fusion mediates angiotensin II-induced lipid raft clustering in mesenteric endothelial cells**
Han, W-Q., Chen, W-D., Zhang, K., Liu, J-J., Wu, Y-J. and Gao, P-J.
Hypertension Res., ***39***, *227-236 (2016)*

It has been reported that intracellular Ca^{2+} is involved in lysosome fusion and membrane repair in skeletal cells. Given that angiotensin II (Ang II) elicits an increase in intracellular Ca^{2+} and that lysosome fusion is a crucial mediator of lipid raft (LR) clustering, we hypothesized that Ang II induces lysosome fusion and activates LR formation in rat mesenteric endothelial cells (MECs). We found that Ang II acutely increased intracellular Ca^{2+} content, an effect that was inhibited by the extracellular Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) and the inositol 1,4,5-trisphosphate (IP3)-induced Ca^{2+} release inhibitor 2-aminoethoxydiphenyl borate (2-APB). Further study showed that EGTA almost completely blocked Ang II-induced lysosome fusion, the translocation of acid sphingomyelinase (ASMase) to LR clusters, ASMase activation and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activation. In contrast, 2-APB had a slight inhibitory effect. Functionally, both the lysosome inhibitor bafilomycin A1 and the ASMase inhibitor amitriptyline reversed Ang II-induced impairment of vasodilation. We conclude that Ca^{2+}-regulated lysosome fusion mediates the Ang II-induced regulation of the LR-redox signaling pathway and mesenteric endothelial dysfunction.

3.2496 **Vaginal epithelial cells regulate membrane adhesiveness to co-ordinate bacterial adhesion**
Younes, J.A., Klappe, K., Kok, J.W., Busscher, H.J., Reid, G. and van der Mei, H.C.
Cell. Microbiol., ***18(4)***, *605-614 (2016)*

Vaginal epithelium is colonized by different bacterial strains and species. The bacterial composition of vaginal biofilms controls the balance between health and disease. Little is known about the relative contribution of the epithelial and bacterial cell surfaces to bacterial adhesion and whether and how adhesion is regulated over cell membrane regions. Here, we show that bacterial adhesion forces with cell membrane regions not located above the nucleus are stronger than with regions above the nucleus both for vaginal pathogens and different commensal and probiotic lactobacillus strains involved in health. Importantly, adhesion force ratios over membrane regions away from and above the nucleus coincided with the ratios between numbers of adhering bacteria over both regions. Bacterial adhesion forces were dramatically decreased by depleting the epithelial cell membrane of cholesterol or sub-membrane cortical actin. Thus, epithelial cells can regulate membrane regions to which bacterial adhesion is discouraged, possibly to protect the nucleus.

3.2497 **Cellular cholesterol accumulation modulates high fat high sucrose (HFHS) diet-induced ER stress and hepatic inflammasome activation in the development of non-alcoholic steatohepatitis**
Bashiri, A., Nesan, D., Tavallaee, G., Sue-Chue-Lam, I., Chien, K., maguire, G.F., Naples, M., Zhang, J., Magomedova, L., Adeli, K., Cummins, C.L. and Ng, D.S.
Biocheim. Biophys. Acta, ***1861****, 594-605 (2016)*

Non-alcoholic steatohepatitis (NASH), is the form of non-alcoholic fatty liver disease posing risk to progress into serious long term complications. Human and pre-clinical models implicate cellular cholesterol dysregulation playing important role in its development. Mouse model studies suggest synergism between dietary cholesterol and fat in contributing to NASH but the mechanisms remain poorly understood. Our laboratory previously reported the primary importance of hepatic endoplasmic reticulum cholesterol (ER-Chol) in regulating hepatic ER stress by comparing the responses of wild type, *Ldlr* −/− *xLcat* +/+ and *Ldlr* −/− *xLcat* −/− mice, to a 2% high cholesterol diet (HCD). Here we further investigated the roles of ER-Chol and ER stress in HFHS diet-induced NASH using the same strains. With HFHS diet feeding, both WT and *Ldlr* −/− *xLcat* +/+ accumulate ER-Chol in association with ER stress and inflammasome activation but the *Ldlr* −/− *xLcat* −/− mice are protected. By contrast, all three strains accumulate cholesterol crystal, in correlation with ER-Chol, albeit less so in *Ldlr* −/− *xLcat* −/− mice. By comparison, HCD feeding per se (i) is sufficient to promote steatosis and activate inflammasomes, and (ii) results in dramatic accumulation of cholesterol crystal which is linked to inflammasome activation in *Ldlr* −/− *xLcat* −/− mice, independent of ER-Chol. Our data suggest that both dietary fat and cholesterol each independently promote steatosis, cholesterol crystal accumulation and inflammasome activation through distinct but complementary pathways. In vitro studies using palmitate-induced hepatic steatosis in HepG2 cells confirm the key roles by cellular cholesterol in the induction of steatosis and inflammasome activations. These novel findings provide opportunities for exploring a cellular cholesterol-focused strategy for treatment of NASH.

3.2498 **Comparing exosome-like vesicles with liposomes for the functional cellular delivery of small RNAs**
Stremersch, S., Vandenbroucke, R.E., Van Wonterghem, E., hendrix, A., De Smedt, S. and Raemdonck, K.
J. Controlled Release, ***232****, 51-61 (2016)*

Exosome-like vesicles (ELVs) play an important role in intercellular communication by acting as natural carriers for biomolecule transfer between cells. This unique feature rationalizes their exploitation as bio-inspired drug delivery systems. However, the therapeutic application of ELVs is hampered by the lack of efficient and reproducible drug loading methods, in particular for therapeutic macromolecules. To overcome this limitation, we present a generic method to attach siRNA to the surface of isolated ELVs by means of a cholesterol anchor. Despite a feasible uptake in both a dendritic and lung epithelial cell line, B16F10- and JAWSII-derived ELVs were unable to functionally deliver the associated small RNAs, neither exogenous cholesterol-conjugated siRNA nor endogenous miRNA derived from the melanoma producer cell. The latter results were confirmed both for purified ELVs and ELVs delivered via a transwell co-culture set-up. In contrast, simple anionic fusogenic liposomes were able to induce a marked siRNA-mediated gene knockdown under equal experimental conditions, both indicating successful cytosolic delivery of surface-bound cholesterol-conjugated siRNA and further underscoring the incapacity of the here evaluated ELVs to guide cytosolic delivery of small RNAs. In conclusion, we demonstrate that a more in-depth understanding of the biomolecular delivery mechanism and specificity is required before ELVs can be envisioned as a generic siRNA carrier.

3.2499 **Identification of TBK1 and IKKε, the non-canonical IκB kinases, as crucial pro-survival factors in HTLV-1-transformed T lymphocytes**
Hang, H., Chen, L., Cai, S-H. and Cheng, H.
Leukemia Res., ***46****, 37-44 (2016)*

Persistent activation of NF-κB is a prerequisite for development of adult T cell leukemia-lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). HTLV-1 genome encodes a viral transforming protein named Tax, which constitutively activates the canonical IκB kinases (IKK), the central regulator of NF-κB signaling. However, the role of the non-canonical IκB kinases, TBK1 and IKKε, in the pathogenesis of HTLV-1-associated leukemia has not been evaluated. We here show that TBK1/IKKε are crucial pro-survival molecules by maintaining persistent activity of Stat3. Consistent with this finding, silencing Stat3 by the specific shRNA or by the chemical inhibitor ruxolitinib results in drastic impediment of leukemia cell growth. We further find that in HTLV-1-transformed T cells expressing Tax, TBK1 co-localizes with the canonical IκB kinases and Tax in the lipid raft microdomains. The wild type Tax, but not

the Tax mutant defective in activating the canonical IKK, promotes the lipid raft translocation of TBK1. This phenomenon correlates with Tax activation of both NF-κB and Stat3. Tax does not interact directly with TBK1/IKKε, and it rather engages a molecular crosstalk between the canonical IKKs and TBK1/IKKε. Our data, therefore, demonstrate a key role of TBK1/IKKε in the survival and proliferation of HTLV-1-transformed T cells and implicate a potential therapy targeting TBK1/IKKε and Stat3 in controlling HTLV-1-mediated oncogenesis.

3.2500 Localization and signaling of GPCRs in lipid rafts
Villar, Van Anthony M., Cuevas, S., Zheng, X. and Jose, P.A.
Methods in Cell Biol., ***132***, *3-23 (2016)*

The understanding of how biological membranes are organized and how they function has evolved. Instead of just serving as a medium in which certain proteins are found, portions of the lipid bilayer have been demonstrated to form specialized platforms that foster the assembly of signaling complexes by providing a microenvironment that is conducive for effective protein–protein interactions. G protein-coupled receptors (GPCRs) and relevant signaling molecules, including the heterotrimeric G proteins, key enzymes such as kinases and phosphatases, trafficking proteins, and secondary messengers, preferentially partition to these highly organized cell membrane microdomains, called lipid rafts. As such, lipid rafts are crucial for the trafficking and signaling of GPCRs. The study of GPCR biology in the context of lipid rafts involves the localization of the GPCR of interest in lipid rafts, at the basal state and upon receptor agonism, and the evaluation of the biological functions of the GPCR in appropriate cell lines. The lack of standardized methodology to study lipid rafts, in general, and of the workings of GPCRs in lipid rafts, in particular, and the inherent drawbacks of current methods have hampered the complete understanding of the underlying molecular mechanisms. Newer methodologies that allow the study of GPCRs in their native form are needed. The use of complementary approaches that produce mutually supportive results appear to be the best way for drawing conclusions with regards to the distribution and activity of GPCRs in lipid rafts.

3.2501 Cytotoxic and Inflammatory Responses Induced by Outer Membrane Vesicle-Associated Biologically Active Proteases from Vibrio cholera
Mondal, A., Tapader, R., Chatterjee, N.S.C., Ghosh, A., Sinha, R., Koley, H., Saha, D.R., Chakrabarti, M.K., Wai, S.N. and Pal, A.
Infect. Immun. ***84(5)***, *1478-1490 (2016)*

Proteases in Vibrio cholerae have been shown to play a role in its pathogenesis. V. cholerae secretes Zn-dependent hemagglutinin protease (HAP) and calcium-dependent trypsin-like serine protease (VesC) by using the type II secretion system (TIISS). Our present studies demonstrated that these proteases are also secreted in association with outer membrane vesicles (OMVs) and transported to human intestinal epithelial cells in an active form. OMV-associated HAP induces dose-dependent apoptosis in Int407 cells and an enterotoxic response in the mouse ileal loop (MIL) assay, whereas OMV-associated VesC showed a hemorrhagic fluid response in the MIL assay, necrosis in Int407 cells, and an increased interleukin-8 (IL-8) response in T84 cells, which were significantly reduced in OMVs from VesC mutant strain. Our results also showed that serine protease VesC plays a role in intestinal colonization of V. cholerae strains in adult mice. In conclusion, our study shows that V. cholerae OMVs secrete biologically active proteases which may play a role in cytotoxic and inflammatory responses.

3.2502 Membrane-association of mRNA decapping factors is independent of stress in budding yeast
Huch, S., Gommlich, J., Muppavarapu, M., Beckham, C. and Nissan, T.
Scientific Reports, ***6****:25477 (2016)*

Recent evidence has suggested that the degradation of mRNA occurs on translating ribosomes or alternatively within RNA granules called P bodies, which are aggregates whose core constituents are mRNA decay proteins and RNA. In this study, we examined the mRNA decapping proteins, Dcp1, Dcp2, and Dhh1, using subcellular fractionation. We found that decapping factors co-sediment in the polysome fraction of a sucrose gradient and do not alter their behaviour with stress, inhibition of translation or inhibition of the P body formation. Importantly, their localisation to the polysome fraction is independent of the RNA, suggesting that these factors may be constitutively localised to the polysome. Conversely, polysomal and post-polysomal sedimentation of the decapping proteins was abolished with the addition of a detergent, which shifts the factors to the non-translating RNP fraction and is consistent with membrane association. Using a membrane flotation assay, we observed the mRNA decapping factors in the lower density fractions at the buoyant density of membrane-associated proteins. These observations provide

further evidence that mRNA decapping factors interact with subcellular membranes, and we suggest a model in which the mRNA decapping factors interact with membranes to facilitate regulation of mRNA degradation.

3.2503 Trehalose Alters Subcellular Trafficking and the Metabolism of the Alzheimer-associated Amyloid Precursor Protein

Tien, N.T., karaca, I., Tamboli, I.Y. and Walter, J.
*J. Biol Chem., **291(20)**, 10528-10540 (2016)*

The disaccharide trehalose is commonly considered to stimulate autophagy. Cell treatment with trehalose could decrease cytosolic aggregates of potentially pathogenic proteins, including mutant huntingtin, α-synuclein, and phosphorylated tau that are associated with neurodegenerative diseases. Here, we demonstrate that trehalose also alters the metabolism of the Alzheimer disease-related amyloid precursor protein (APP). Cell treatment with trehalose decreased the degradation of full-length APP and its C-terminal fragments. Trehalose also reduced the secretion of the amyloid-β peptide. Biochemical and cell biological experiments revealed that trehalose alters the subcellular distribution and decreases the degradation of APP C-terminal fragments in endolysosomal compartments. Trehalose also led to strong accumulation of the autophagic marker proteins LC3-II and p62, and decreased the proteolytic activation of the lysosomal hydrolase cathepsin D. The combined data indicate that trehalose decreases the lysosomal metabolism of APP by altering its endocytic vesicular transport.

3.2504 MAL Is a Regulator of the Recruitment of Myelin Protein PLP to Membrane Microdomains

Bijlard, M., de Jonge, J.C., Klunder, B., Nomden, A., Hoekstra, D. and Baron, W.
*PloS One, **11(5)**, e0155317 (2016)*

In oligodendrocytes (OLGs), an indirect, transcytotic pathway is mediating transport of de novo synthesized PLP, a major myelin specific protein, from the apical-like plasma membrane to the specialized basolateral-like myelin membrane to prevent its premature compaction. MAL is a well-known regulator of polarized trafficking in epithelial cells, and given its presence in OLGs it was therefore of interest to investigate whether MAL played a similar role in PLP transport in OLGs, taking into account its timely expression in these cells. Our data revealed that premature expression of mCherry-MAL in oligodendrocyte progenitor cells interfered with terminal OLG differentiation, although myelin membrane formation per se was not impaired. In fact, also PLP transport to myelin membranes via the cell body plasma membrane was unaffected. However, the typical shift of PLP from TX-100-insoluble membrane domains to CHAPS-resistant, but TX-100-soluble membrane domains, seen in the absence of MAL expression, is substantially reduced upon expression of the MAL protein. Interestingly, not only in vitro, but also in developing brain a strongly diminished shift from TX-100 resistant to TX-100 soluble domains was observed. Consistently, the MAL-expression mediated annihilation of the typical membrane microdomain shift of PLP is also reflected by a loss of the characteristic surface expression profile of conformation-sensitive anti-PLP antibodies. Hence, these findings suggest that MAL is not involved in vesicular PLP trafficking to either the plasma membrane and/or the myelin membrane as such. Rather, we propose that MAL may regulate PLP's distribution into distinct membrane microdomains that allow for lateral diffusion of PLP, directly from the plasma membrane to the myelin membrane once the myelin sheath has been assembled.

3.2505 Blood-Based Biomarkers for Metabolic Syndrome

O'Neill, S., Bphl, M., Gregersen, S., Hermansen, K. and O'Driscoll, L.
*Trends in Endocrinology & Metabolism, **27(6)**, 363-374 (2016)*

Metabolic syndrome (MetS) is a constellation of factors increasing the risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and cancer. MetS diagnosis is cumbersome and the precise diagnosis differs throughout the world. Efforts are underway to find MetS biomarkers that could all be analysed in a single blood sample. Here we review recent advances, including progress on circulating exosomes and microvesicles and their molecular contents, as well as DNA, RNAs, and proteins taken directly from blood samples. While additional research is now warranted to advance upon these findings, there is reason for optimising that such blood-based entities will be beneficial for MetS diagnosis and will help reduce risk of T2DM, CVD, and cancers, contributing both societal and economic benefit.

3.2506 ABCG1 and ABCG4 Suppress γ-Secretase Activity and Amyloid β Production
Sano, O., Tsujita, M., Shimizu, Y., Kato, R., Kobayashi, A., Kioka, N., Remaley, A.T., Michikawa, M., Ueda, K. and Matsuo, M.
*PloS One, **11(5)**, e0155400 (2016)*

ATP-binding cassette G1 (ABCG1) and ABCG4, expressed in neurons and glia in the central nervous system, mediate cholesterol efflux to lipid acceptors. The relationship between cholesterol level in the central nervous system and Alzheimer's disease has been reported. In this study, we examined the effects of ABCG1 and ABCG4 on amyloid precursor protein (APP) processing, the product of which, amyloid β (Aβ), is involved in the pathogenesis of Alzheimer's disease. Expression of ABCG1 or ABCG4 in human embryonic kidney 293 cells that stably expressed Swedish-type mutant APP increased cellular and cell surface APP levels. Products of cleavage from APP by α-secretase and by β-secretase also increased. The levels of secreted Aβ, however, decreased in the presence of ABCG1 and ABCG4, but not ABCG4-KM, a nonfunctional Walker-A lysine mutant. In contrast, secreted Aβ levels increased in differentiated SH-SY5Y neuron-like cells in which ABCG1 and ABCG4 were suppressed. Furthermore, Aβ42 peptide in the cerebrospinal fluid from Abcg1 null mice significantly increased compared to the wild type mice. To examine the underlying mechanism, we analyzed the activity and distribution of γ-secretase. ABCG1 and ABCG4 suppressed γ-secretase activity and disturbed γ-secretase localization in the raft domains where γ-secretase functions. These results suggest that ABCG1 and ABCG4 alter the distribution of γ-secretase on the plasma membrane, leading to the decreased γ-secretase activity and suppressed Aβ secretion. ABCG1 and ABCG4 may inhibit the development of Alzheimer's disease and can be targets for the treatment of Alzheimer's disease.

3.2507 Do Src Kinase and Caveolin Interact Directly with Na,K-ATPase?
Yosef, E., Katz, A., Peleg, Y., Mehlman, T. and Karlish, J.D:
*J. Biol. Chem., **291(22)**, 11736-11750 (2016)*

Much evidence points to a role of Na,K-ATPase in ouabain-dependent signal transduction. Based on experiments with different cell lines and native tissue membranes, a current hypothesis postulates direct interactions between the Na,K-ATPase and Src kinase (non-receptor tyrosine kinase). Na,K-ATPase is proposed to bind Src kinase and inhibit its activity, whereas ouabain, the specific Na,K-ATPase inhibitor, binds and stabilizes the E2 conformation, thus exposing the Src kinase domain and its active site Tyr-418 for activation. Ouabain-dependent signaling is thought to be mediated within caveolae by a complex consisting of Na,K-ATPase, caveolin, and Src kinase. In the current work, we have looked for direct interactions utilizing purified recombinant Na,K-ATPase (human α1β1FXYD1 or porcine α1D369Nβ1FXYD1) and purified human Src kinase and human caveolin 1 or interactions between these proteins in native membrane vesicles isolated from rabbit kidney. By several independent criteria and techniques, no stable interactions were detected between Na,K-ATPase and purified Src kinase. Na,K-ATPase was found to be a substrate for Src kinase phosphorylation at Tyr-144. Clear evidence for a direct interaction between purified human Na,K-ATPase and human caveolin was obtained, albeit with a low molar stoichiometry (1:15–30 caveolin 1/Na,K-ATPase). In native renal membranes, a specific caveolin 1_{4-5} oligomer (95 kDa) was found to be in direct interaction with Na,K-ATPase. We inferred that a small fraction of the renal Na,K-ATPase molecules is in a ~1:1 complex with a caveolin 1_{4-5} oligomer. Thus, overall, whereas a direct caveolin 1/Na,K-ATPase interaction is confirmed, the lack of direct Src kinase/Na,K-ATPase binding requires reassessment of the mechanism of ouabain-dependent signaling.

3.2508 Preparation of Gap Junctions in Membrane Microdomains for Immunoprecipitation and Mass Spectrometry Interactome Analysis
Fowler, S., Akins, M. and Bennett, S.A.L.
*Methods in Mol. Biol., **1437**, 113-132 (2016)*

Protein interaction networks at gap junction plaques are increasingly implicated in a variety of intracellular signaling cascades. Identifying protein interactions of integral membrane proteins is a valuable tool for determining channel function. However, several technical challenges exist. Subcellular fractionation of the bait protein matrix is usually required to identify less abundant proteins in complex homogenates. Sufficient solvation of the lipid environment without perturbation of the protein interactome must also be achieved. The present chapter describes the flotation of light and heavy liver tissue membrane microdomains to facilitate the identification and analysis of endogenous gap junction proteins and includes technical notes for translation to other integral membrane proteins, tissues, or cell culture models. These

procedures are valuable tools for the enrichment of gap junction membrane compartments and for the identification of gap junction signaling interactomes.

3.2509 Lysosomal cholesterol accumulation in macrophages leading to coronary atherosclerosis in CD38−/− mice

Xu, X., Yuan, X., Li, N., Dewey, W.L., Li, P-L. and Zhang, F.
*J. Cell. Mol. Med., **20(6)**, 1001-1013 (2016)*

The disruption in transportation of oxLDL-derived cholesterol and the subsequent lipid accumulation in macrophages are the hallmark events in atherogenesis. Our recent studies demonstrated that lysosomal Ca^{2+} messenger of nicotinic acid adenine dinucleotide phosphate (NAADP), an enzymatic product of CD38 ADP-ribosylcyclase (CD38), promoted lipid endocytic trafficking in human fibroblast cells. The current studies are designed to examine the functional role of CD38/NAADP pathway in the regulation of lysosomal cholesterol efflux in atherosclerosis. Oil red O staining showed that oxLDL concentration-dependently increased lipid buildup in bone marrow-derived macrophages from both wild type and $CD38^{-/-}$, but to a significant higher extent with CD38 gene deletion. Bodipy 493/503 fluorescence staining found that the deposited lipid in macrophages was mainly enclosed in lysosomal organelles and largely enhanced with the blockade of CD38/NAADP pathway. Filipin staining and direct measurement of lysosome fraction further revealed that the free cholesterol constituted a major portion of the total cholesterol segregated in lysosomes. Moreover, *in situ* assay disclosed that both lysosomal lumen acidity and the acid lipase activity were reduced upon cholesterol buildup in lysosomes. In $CD38^{-/-}$ mice, treatment with Western diet (12 weeks) produced atherosclerotic damage in coronary artery with striking lysosomal cholesterol sequestration in macrophages. These data provide the first experimental evidence that the proper function of CD38/NAADP pathway plays an essential role in promoting free cholesterol efflux from lysosomes and that a defection of this signalling leads to lysosomal cholesterol accumulation in macrophages and results in coronary atherosclerosis in $CD38^{-/-}$ mice.

3.2510 Demonstration of an oligosaccharide-diphosphodolichol diphosphatase activity whose subcellular localization is different than those of dolichyl-phosphate-dependent enzymes of the dolichol cycle

Massarweh, A., Bosco, M., iatmanen-harbi, S., tessier, C., Auberger, N., Busca, P., Chantret, I., Gravier-Pelletier, C. and Moore, S.E.H.
*J. Lipid Res., **57**, 1029-1042 (2016)*

Oligosaccharyl phosphates (OSPs) are hydrolyzed from oligosaccharide-diphosphodolichol (DLO) during protein *N*-glycosylation by an uncharacterized process. An OSP-generating activity has been reported in vitro, and here we asked if its biochemical characteristics are compatible with a role in endoplasmic reticulum (ER)-situated DLO regulation. We demonstrate a Co^{2+}-dependent DLO diphosphatase (DLODP) activity that splits DLO into dolichyl phosphate and OSP. DLODP has a pH optimum of 5.5 and is inhibited by vanadate but not by NaF. Polyprenyl diphosphates inhibit [^{3}H]OSP release from [^{3}H]DLO, the length of their alkyl chains correlating positively with inhibition potency. The diphosphodiester $GlcNAc_2$-PP-solanesol is hydrolyzed to yield $GlcNAc_2$-P and inhibits [^{3}H]OSP release from [^{3}H]DLO more effectively than the diphosphomonoester solanesyl diphosphate. During subcellular fractionation of liver homogenates, DLODP codistributes with microsomal markers, and density gradient centrifugation revealed that the distribution of DLODP is closer to that of Golgi apparatus-situated UDP-galactose glycoprotein galactosyltransferase than those of dolichyl-P-dependent glycosyltransferases required for DLO biosynthesis in the ER. Therefore, a DLODP activity showing selectivity toward lipophilic diphosphodiesters such as DLO, and possessing properties distinct from other lipid phosphatases, is identified. Separate subcellular locations for DLODP action and DLO biosynthesis may be required to prevent uncontrolled DLO destruction.

3.2511 Phosphatidylinositol-3-phosphate is light-regulated and essential for survival in retinal rods

He, F., Agosto, M.A., Anastassov, I.A., Tse, D.Y., Wu, S.M. and Wensel, T.G.
*Scientific Reports, **6**:26978 (2016)*

Phosphoinositides play important roles in numerous intracellular membrane pathways. Little is known about the regulation or function of these lipids in rod photoreceptor cells, which have highly active membrane dynamics. Using new assays with femtomole sensitivity, we determined that whereas levels of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate were below detection limits, phosphatidylinositol-3-phosphate (PI(3)P) levels in rod inner/outer segments increased more than 30-fold after light exposure. This increase was blocked in a rod-specific knockout of the PI-3 kinase Vps34,

resulting in failure of endosomal and autophagy-related membranes to fuse with lysosomes, and accumulation of abnormal membrane structures. At early ages, rods displayed normal morphology, rhodopsin trafficking, and light responses, but underwent progressive neurodegeneration with eventual loss of both rods and cones by twelve weeks. The degeneration is considerably faster than in rod knockouts of autophagy genes, indicating defects in endosome recycling or other PI(3)P-dependent membrane trafficking pathways are also essential for rod survival.

3.2512 Adjuvant-Loaded Subcellular Vesicles Derived From Disrupted Cancer Cells for Cancer Vaccination
Cheung, A.S., Koshy, S.T., Stafford, A.G., Bastings, M.M.C. and Mooney, D.J.
*Small, **12(17)**, 2321-2333 (2016)*

Targeted subunit vaccines for cancer immunotherapy do not capture tumor antigenic complexity, and approaches employing tumor lysate are often limited by inefficient antigen uptake and presentation, and low immunogenicity. Here, whole cancer cells are processed to generate antigen-rich, membrane-enclosed subcellular particles, termed “reduced cancer cells”, that reflect the diversity and breadth of the parent cancer cell antigen repertoire, and can be loaded with disparate adjuvant payloads. These vesicular particles enhance the uptake of the adjuvant payload, and potentiate the activation of primary dendritic cells in vitro. Similarly, reduced cancer cell-associated antigens are more efficiently presented by primary dendritic cells in vitro than their soluble counterparts or lysate control. In mice, vaccination using adjuvant-loaded reduced cancer cells facilitates the induction of antigen-specific cellular and humoral immune responses. Taken together, these observations demonstrate that adjuvant-loaded reduced cancer cells could be utilized in cancer vaccines as an alternative to lysate.

3.2513 Enhancement of β-catenin activity by BIG1 plus BIG2 via Arf activation and cAMP signals
Li, C-C., Le, K., kato, J., Moss, J. and Vaughan, M.
*PNAS, **113(21)**, 5946-5941 (2016)*

Multifunctional β-catenin, with critical roles in both cell–cell adhesion and Wnt-signaling pathways, was among HeLa cell proteins coimmunoprecipitated by antibodies against brefeldin A-inhibited guanine nucleotide-exchange factors 1 and 2 (BIG1 or BIG2) that activate ADP-ribosylation factors (Arfs) by accelerating the replacement of bound GDP with GTP. BIG proteins also contain A-kinase anchoring protein (AKAP) sequences that can act as scaffolds for multimolecular assemblies that facilitate and limit cAMP signaling temporally and spatially. Direct interaction of BIG1 N-terminal sequence with β-catenin was confirmed using yeast two-hybrid assays and in vitro synthesized proteins. Depletion of BIG1 and/or BIG2 or overexpression of guanine nucleotide-exchange factor inactive mutant, but not wild-type, proteins interfered with β-catenin trafficking, leading to accumulation at perinuclear Golgi structures. Both phospholipase D activity and vesicular trafficking were required for effects of BIG1 and BIG2 on β-catenin activation. Levels of PKA-phosphorylated β-catenin S675 and β-catenin association with PKA, BIG1, and BIG2 were also diminished after BIG1/BIG2 depletion. Inferring a requirement for BIG1 and/or BIG2 AKAP sequence in PKA modification of β-catenin and its effect on transcription activation, we confirmed dependence of S675 phosphorylation and transcription coactivator function on BIG2 AKAP-C sequence.

3.2514 Extracellular vesicles in cardiovascular disease: are they Jedi or Sith?
Osteikoetxea, X., Nemeth, A., Sodar, B.W., Vukman, K.V. and Buzas, E.I.
*J. Physiol., **594(11)**, 2881-2894 (2016)*

In the recent past, extracellular vesicles have become recognized as important players in cell biology and biomedicine. Extracellular vesicles, including exosomes, microvesicles and apoptotic bodies, are phospholipid bilayer-enclosed structures found to be secreted by most if not all cells. Extracellular vesicle secretion represents a universal and highly conserved active cellular function. Importantly, increasing evidence supports that extracellular vesicles may serve as biomarkers and therapeutic targets or tools in human diseases. Cardiovascular disease undoubtedly represents one of the most intensely studied and rapidly growing areas of the extracellular vesicle field. However, in different studies related to cardiovascular disease, extracellular vesicles have been shown to exert diverse and sometimes discordant biological effects. Therefore, it might seem a puzzle whether these vesicles are in fact beneficial or detrimental to cardiovascular health. In this review we provide a general introduction to extracellular vesicles and an overview of their biological roles in cardiovascular diseases. Furthermore, we aim to untangle the various reasons for the observed discrepancy in biological effects of extracellular vesicles in

cardiovascular diseases. To this end, we provide several examples that demonstrate that the observed functional diversity is in fact due to inherent differences among various types of extracellular vesicles.

3.2515 Sequential steps of macroautophagy and chaperone-mediated autophagy are involved in the irreversible process of posterior silk gland histolysis during metamorphosis of Bombyx mori
Shiba, H., yabu, T., Sudayama, M., Mano, N., Arai, N., Nakanishi, T. and Hosono, K.
J. Exp. Biol., ***219****, 1146-1153 (2016)*

To elucidate the degradation process of the posterior silk gland during metamorphosis of the silkworm *Bombyx mori*, tissues collected on the 6th day after entering the 5th instar (V6), prior to spinning (PS), during spinning (SP) and after cocoon formation (CO) were used to analyze macroautophagy, chaperone-mediated autophagy (CMA) and the adenosine triphosphate (ATP)-dependent ubiquitin proteasome. Immediately after entering metamorphosis stage PS, the levels of ATP and phosphorylated p70S6 kinase protein decreased spontaneously and continued to decline at SP, followed by a notable restoration at CO. In contrast, phosphorylated AMP-activated protein kinase α (AMPKα) showed increases at SP and CO. Most of the Atg8 protein was converted to form II at all stages. The levels of ubiquitinated proteins were high at SP and CO, and low at PS. The proteasome activity was high at V6 and PS but low at SP and CO. In the isolated lysosome fractions, levels of Hsc70/Hsp70 protein began to increase at PS and continued to rise at SP and CO. The lysosomal cathepsin B/L activity showed a dramatic increase at CO. Our results clearly demonstrate that macroautophagy occurs before entering the metamorphosis stage and strongly suggest that the CMA pathway may play an important role in the histolysis of the posterior silk gland during metamorphosis

3.2516 Reciprocal regulation of actin cytoskeleton remodelling and cell migration by Ca2+ and Zn2+: role of TRPM2 channels
Li, F., Abuarab, N. and Sivaprasadarao, A.
J. Cell Sci., ***129(10)****, 2016-2029 (2016)*

Cell migration is a fundamental feature of tumour metastasis and angiogenesis. It is regulated by a variety of signalling molecules including H_2O_2 and Ca^{2+}. Here, we asked whether the H_2O_2-sensitive transient receptor potential melastatin 2 (TRPM2) Ca^{2+} channel serves as a molecular link between H_2O_2 and Ca^{2+}. H_2O_2-mediated activation of TRPM2 channels induced filopodia formation, loss of actin stress fibres and disassembly of focal adhesions, leading to increased migration of HeLa and prostate cancer (PC)-3 cells. Activation of TRPM2 channels, however, caused intracellular release of not only Ca^{2+} but also of Zn^{2+}. Intriguingly, elevation of intracellular Zn^{2+} faithfully reproduced all of the effects of H_2O_2, whereas Ca^{2+} showed opposite effects. Interestingly, H_2O_2 caused increased trafficking of Zn^{2+}-enriched lysosomes to the leading edge of migrating cells, presumably to impart polarisation of Zn^{2+} location. Thus, our results indicate that a reciprocal interplay between Ca^{2+} and Zn^{2+} regulates actin remodelling and cell migration; they call for a revision of the current notion that implicates an exclusive role for Ca^{2+} in cell migration.

3.2517 Heparin interacts with the adhesion GPCR GPR56, reduces receptor shedding, and promotes cell adhesion and motility
Chiang, N-Y., Chang, G-W., Huang, Y-S., Peng, Y-M., Hsiao, C-C., Kuo, M-L. and Lin, H-H.
J. Cell Sci., ***129(11)****, 2156-2169 (2016)*

GPR56 is an adhesion-class G-protein-coupled receptor responsible for bilateral frontoparietal polymicrogyria (BFPP), a severe disorder of cortical formation. Additionally, GPR56 is involved in biological processes as diverse as hematopoietic stem cell generation and maintenance, myoblast fusion, muscle hypertrophy, immunoregulation and tumorigenesis. Collagen III and tissue transglutaminase 2 (TG2) have been revealed as the matricellular ligands of GPR56 involved in BFPP and melanoma development, respectively. In this study, we identify heparin as a glycosaminoglycan interacting partner of GPR56. Analyses of truncated and mutant GPR56 proteins reveal two basic-residue-rich clusters, R^{26}GHREDFRFC^{35} and L^{190}KHPQKASRRP^{200}, as the major heparin-interacting motifs that overlap partially with the collagen III- and TG2-binding sites. Interestingly, the GPR56–heparin interaction is modulated by collagen III but not TG2, even though both ligands are also heparin-binding proteins. Finally, we show that the interaction with heparin reduces GPR56 receptor shedding, and enhances cell adhesion and motility. These results provide novel insights into the interaction of GPR56 with its multiple endogenous ligands and have functional implications in diseases such as BFPP and cancer

3.2518 Kinesin 1 Drives Autolysosome Tubulation
Du, W., Su, Q.P., Chen, Y. et al
*Developmental Cell, **37(4)**, 326-336 (2016)*

Autophagic lysosome reformation (ALR) plays an important role in maintaining lysosome homeostasis. During ALR, lysosomes are reformed by recycling lysosomal components from autolysosomes. The most noticeable step of ALR is autolysosome tubulation, but it is currently unknown how the process is regulated. Here, using an approach combining in vivo studies and in vitro reconstitution, we found that the kinesin motor protein KIF5B is required for autolysosome tubulation and that KIF5B drives autolysosome tubulation by pulling on the autolysosomal membrane. Furthermore, we show that KIF5B directly interacts with PtdIns(4,5)P_2. Kinesin motors are recruited and clustered on autolysosomes via interaction with PtdIns(4,5)P_2 in a clathrin-dependent manner. Finally, we demonstrate that clathrin promotes formation of PtdIns(4,5)P_2-enriched microdomains, which are required for clustering of KIF5B. Our study reveals a mechanism by which autolysosome tubulation was generated.

3.2519 Plasticity of sarcolemmal KATP channel surface expression: relevance during ischemia and ischemic preconditioning
Yang, H-Q., Foster, M.N., jana, K., Ho, J., Rindler, M.J. and Coetzee, W.A.
*Am. J. Physiol. Heart Circ. Physiol., **310(11)**, H1558-H1566 (2016)*

Myocardial ischemia remains the primary cause of morbidity and mortality in the United States. Ischemic preconditioning (IPC) is a powerful form of endogenous protection against myocardial infarction. We studied alterations in K_{ATP} channels surface density as a potential mechanism of the protection of IPC. Using cardiac-specific knockout of Kir6.2 subunits, we demonstrated an essential role for sarcolemmal K_{ATP} channels in the infarct-limiting effect of IPC in the mouse heart. With biochemical membrane fractionation, we demonstrated that sarcolemmal K_{ATP} channel subunits are distributed both to the sarcolemma and intracellular endosomal compartments. Global ischemia causes a loss of sarcolemmal K_{ATP} channel subunit distribution and internalization to endosomal compartments. Ischemia-induced internalization of K_{ATP} channels was prevented by CaMKII inhibition. K_{ATP} channel subcellular redistribution was also observed with immunohistochemistry. Ischemic preconditioning before the index ischemia reduced not only the infarct size but also prevented K_{ATP} channel internalization. Furthermore, not only did adenosine mimic IPC by preventing infarct size, but it also prevented ischemia-induced K_{ATP} channel internalization via a PKC-mediated pathway. We show that preventing endocytosis with dynasore reduced both K_{ATP} channel internalization and strongly mitigated infarct development. Our data demonstrate that plasticity of K_{ATP} channel surface expression must be considered as a potentially important mechanism of the protective effects of IPC and adenosine

3.2520 Control of chylomicron export from the intestine
Mansbach II, C.M. and Siddiqi, S.
*Am. J. Gastrointest. Liver Physiol., **310(9)**, G659-G668 (2016)*

The control of chylomicron output by the intestine is a complex process whose outlines have only recently come into focus. In this review we will cover aspects of chylomicron formation and prechylomicron vesicle generation that elucidate potential control points. Substrate (dietary fatty acids and monoacylglycerols) availability is directly related to the output rate of chylomicrons. These substrates must be converted to triacylglycerol before packaging in prechylomicrons by a series of endoplasmic reticulum (ER)-localized acylating enzymes that rapidly convert fatty acids and monoacylglycerols to triacylglycerol. The packaging of the prechylomicron with triacylglycerol is controlled by the microsomal triglyceride transport protein, another potential limiting step. The prechylomicrons, once loaded with triacylglycerol, are ready to be incorporated into the prechylomicron transport vesicle that transports the prechylomicron from the ER to the Golgi. Control of this exit step from the ER, the rate-limiting step in the transcellular movement of the triacylglycerol, is a multistep process involving the activation of PKCζ, the phosphorylation of Sar1b, releasing the liver fatty acid binding protein from a heteroquatromeric complex, which enables it to bind to the ER and organize the prechylomicron transport vesicle budding complex. We propose that control of PKCζ activation is the major physiological regulator of chylomicron output.

3.2521 GPRC5A suppresses protein synthesis at the endoplasmic reticulum to prevent radiation-induced lung tumorigenesis

Wang, J., Farris, A.B., Xu, K., Wang, P., Zhang, X., duong, D.M., Yi, H., Shu, H-K., Sun, S-Y. and Wang, Y.

Nature Communications, 7:11795 (2016)

GPRC5A functions as a lung tumour suppressor to prevent spontaneous and environmentally induced lung carcinogenesis; however, the underlying mechanism remains unclear. Here we reveal that GPRC5A at the endoplasmic reticulum (ER) membrane suppresses synthesis of the secreted or membrane-bound proteins including a number of oncogenes, the most important one being *Egfr*. The ER-located GPRC5A disturbs the assembly of the eIF4F-mediated translation initiation complex on the mRNA cap through directly binding to the eIF4F complex with its two middle extracellular loops. Particularly, suppression of EGFR by GPRC5A contributes significantly to preventing ionizing radiation (IR)-induced lung tumorigenesis. Thus, GPRC5A deletion enhances IR-promoted EGFR expression through an increased translation rate, thereby significantly increasing lung tumour incidence in *Gprc5a*$^{-/-}$ mice. Our findings indicate that under-expressed GPRC5A during lung tumorigenesis enhances any transcriptional stimulation through an active translational status, which can be used to control oncogene expression and potentially the resulting related disease.

3.2522 Golgi-localized STELLO proteins regulate the assembly and trafficking of cellulose synthase complexes in Arabidopsis

Zhang, Y. et al

*Nature Communications, **7**:11656 (2016)*

As the most abundant biopolymer on Earth, cellulose is a key structural component of the plant cell wall. Cellulose is produced at the plasma membrane by cellulose synthase (CesA) complexes (CSCs), which are assembled in the endomembrane system and trafficked to the plasma membrane. While several proteins that affect CesA activity have been identified, components that regulate CSC assembly and trafficking remain unknown. Here we show that STELLO1 and 2 are Golgi-localized proteins that can interact with CesAs and control cellulose quantity. In the absence of STELLO function, the spatial distribution within the Golgi, secretion and activity of the CSCs are impaired indicating a central role of the STELLO proteins in CSC assembly. Point mutations in the predicted catalytic domains of the STELLO proteins indicate that they are glycosyltransferases facing the Golgi lumen. Hence, we have uncovered proteins that regulate CSC assembly in the plant Golgi apparatus.

3.2523 Cross-talk between Dopachrome Tautomerase and Caveolin-1 Is Melanoma Cell Phenotype-specific and Potentially Involved in Tumor Progression

Popa, I.L., Milac, A.L., Sima, L.E., Alexandru, P.R., Pastrami, F., Munteanu, C.V.A. and Negroiu, G.

*J. Biol. Cehm., **291(24)**, 12481-12500 (2016)*

l-Dopachrome tautomerase (l-DCT), also called tyrosinase-related protein-2 (TRP-2), is a melanoma antigen overexpressed in most chemo-/radiotherapeutic stress-resistant tumor clones, and caveolin-1 (CAV1) is a main regulator of numerous signaling processes. A structural and functional relationship between DCT and CAV1 is first presented here in two human amelanotic melanoma cell lines, derived from vertical growth phase (MelJuSo) and metastatic (SKMel28) melanomas. DCT co-localizes at the plasma membrane with CAV1 and Cavin-1, another molecular marker for caveolae in both cell phenotypes. Our novel structural model proposed for the DCT-CAV1 complex, in addition to co-immunoprecipitation and mass spectrometry data, indicates a possible direct interaction between DCT and CAV1. The CAV1 control on *DCT* gene expression, DCT post-translational processing, and subcellular distribution is cell phenotype-dependent. DCT is a modulator of CAV1 stability and supramolecular assembly in both cell phenotypes. During autocrine stimulation, the expressions of DCT and CAV1 are oppositely regulated; DCT increases while CAV1 decreases. Sub-confluent MelJuSo clones DCT^{high}/CAV1^{low} are proliferating and acquire fibroblast-like morphology, forming massive, confluent clusters as demonstrated by immunofluorescent staining and TissueFAXS quantitative image cytometry analysis. CAV1 down-regulation directly contributes to the expansion of MelJuSo DCT^{high} subtype. CAV1 involved in the perpetuation of cell phenotype-overexpressing anti-stress DCT molecule supports the concept that CAV1 functions as a tumor suppressor in early stages of melanoma. DCT is a regulator of the CAV1-associated structures.

3.2524 **KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes**

McKenzie, A.J., Hoshino, D., Hong, N.H., Cha, D.J., Franklin, J.F., Coffey, R.J., Patton, J.G. and Weaver, A.M.

*Cell Reports, **15**, 978-987 (2016)*

Secretion of RNAs in extracellular vesicles is a newly recognized form of intercellular communication. A potential regulatory protein for microRNA (miRNA) secretion is the critical RNA-induced silencing complex (RISC) component Argonaute 2 (Ago2). Here, we use isogenic colon cancer cell lines to show that overactivity of KRAS due to mutation inhibits localization of Ago2 to multivesicular endosomes (MVEs) and decreases Ago2 secretion in exosomes. Mechanistically, inhibition of mitogen-activated protein kinase kinases (MEKs) I and II, but not Akt, reverses the effect of the activating KRAS mutation and leads to increased Ago2-MVE association and increased exosomal secretion of Ago2. Analysis of cells expressing mutant Ago2 constructs revealed that phosphorylation of Ago2 on serine 387 prevents Ago2-MVE interactions and reduces Ago2 secretion into exosomes. Furthermore, regulation of Ago2 exosomal sorting controls the levels of three candidate miRNAs in exosomes. These data identify a key regulatory signaling event that controls Ago2 secretion in exosomes.

3.2525 **FLCN Maintains the Leucine Level in Lysosome to Stimulate mTORC1**

Wu, X., Zhao, L., Xhen, Z., Ji, X., Qiao, X., Jin, Y. and Liu, W.

*PloS One, **11(6)**, e0157100 (2016)*

The intracellular amino acid pool within lysosome is a signal that stimulates the nutrient-sensing mTORC1 signalling pathway. The signal transduction cascade has garnered much attention, but little is known about the sequestration of the signalling molecules within the lysosome. Using human HEK293 cells as a model, we found that suppression of the BHD syndrome gene FLCN reduced the leucine level in lysosome, which correlated with decreased mTORC1 activity. Both consequences could be reversed by supplementation with high levels of leucine, but not other tested amino acids. Conversely, overexpressed FLCN could sequester lysosomal leucine and stimulate mTORC1 in an amino acid limitation environment. These results identify a novel function of FLCN: it controls mTORC1 by modulating the leucine signal in lysosome. Furthermore, we provided evidence that FLCN exerted this role by inhibiting the accumulation of the amino acid transporter PAT1 on the lysosome surface, thereby maintaining the signal level within the organelle.

3.2526 **DIETARY FATTY ACID UTILIZES THE CAVEOLIN-1 CONTAINING ENDOCYTIC VESICLES (CEV) AS A VEHICLE FOR TRANSPORT INTO THE INTESTINAL ENDOPLASMIC RETICULUM (ER)**

Morris, L., Siddiqi, T., mansbach, C.M.and Siddiqi, S.

*J. Investig. Med., **64**, abstract 404, 488-726 (2016)*

Purpose of Study How dietary fatty acids (FA) are transported to the ER is not well established. Here we tested the hypothesis that the caveolin-1 containing endocytic vesicle
(CEV) that we proposed as the major mechanism for dietary fatty acid absorption (BBA 183; 1311, 2013) also transports fatty acids into the intestinal endoplasmic reticulum (ER).
Methods Used Native ER and 3H-cytosol were obtained from the enterocytes of wild type (WT) and caveolin-1 (Cav-1) knockout mice. CEV were isolated from 1% Triton X-100 treated cytosol using an OptiPrep gradient, known to be able to separate detergent resistant membranes (DRM) from detergent soluble membranes (DSM). An in vitro binding assay was performed using these fractions with native ER, and then re-isolated ER was analyzed for lipid and protein content.
Summary of Results In WT cytosol, 60% of 3H oleate appeared in the CEV, while 20% was associated with DSM. In Cav-1 KO cytosol, no 3H oleate was found in DRM, whereas 54% of 3H oleate was associated with DSM. When WT ER was incubated with CEV, 68% of the 3H oleate was transported to the ER; greater than the ER incubated with whole cytosol (36%) and DSM (17%). In the Cav-1 KO mice, 21% of 3H oleate was transferred to the ER when incubated with whole cytosol or DSM. Dietary
lipid analysis of the re-isolated ER with both genotypes showed that 89% of the FA had been metabolized into triacyl glycerol (TAG) and diacyl glycerol (DAG) by the end of the 15 min incubations. The amount of Caveolin-1 (Cav-1) and (fatty acid translocase) CD36 showed incremental increases in the ER after binding with CEV; while liver fatty acid binding protein (FABP1) was not increased.

Conclusions The most dietary oleate is absorbed by associating with caveolae in apical BB. The caveolae are endocytosed and appear in cytosol as CEV (BBA 183; 1311, 2013). We conclude that CEVs deliver the dietary FA to the intestinal ER for esterification to TAG, independentof FABP.

3.2527 Identification of Sirtuin4 (SIRT4) Protein Interactions: Uncovering Candidate Acyl-Modified Mitochondrial Substrates and Enzymatic Regulators

Mathias, R.A., Greco, T.M. and Cristea, I.M.
*Methods in Mol. Biol., **1436**, 213-239 (2016)*

Recent studies have highlighted the three mitochondrial human sirtuins (SIRT3, SIRT4, and SIRT5) as critical regulators of a wide range of cellular metabolic pathways. A key factor to understanding their impact on metabolism has been the discovery that, in addition to their ability to deacetylate substrates, mitochondrial sirtuins can have other prominent enzymatic activities. SIRT4, one of the least characterized mitochondrial sirtuins, was shown to be the first known cellular lipoamidase, removing lipoyl modifications from lysine residues of substrates. Specifically, SIRT4 was found to delipoylate and modulate the activity of the pyruvate dehydrogenase complex (PDH), a protein complex critical for the production of acetyl-CoA. Furthermore, SIRT4 is well known to have ADP-ribosyltransferase activity and to regulate the activity of the glutamate dehydrogenase complex (GDH). Adding to its impressive range of enzymatic activities are its ability to deacetylate malonyl-CoA decarboxylase (MCD) to regulate lipid catabolism, and its newly recognized ability to remove biotinyl groups from substrates that remain to be defined. Given the wide range of enzymatic activities and the still limited knowledge of its substrates, further studies are needed to characterize its protein interactions and its impact on metabolic pathways. Here, we present several proven protocols for identifying SIRT4 protein interaction networks within the mitochondria. Specifically, we describe methods for generating human cell lines expressing SIRT4, purifying mitochondria from crude organelles, and effectively capturing SIRT4 with its interactions and substrates.

3.2528 Shaping the endoplasmic reticulum in vitro

Ferencz, C-M., Guigas, G., Veres, A., Neumann, B., Stemmann, O. and Weiss, M.
*Biochim. Biophys. Acta, **1858**, 2035-2040 (2016)*

Organelles in eukaryotic cells often have complex shapes that deviate significantly from simple spheres. A prime example is the endoplasmic reticulum (ER) that forms an extensive network of membrane tubules in many mammalian cell types and in reconstitution assays in vitro. Despite the successful hunt for molecular determinants of ER shape we are still far from having a comprehensive understanding of ER network morphogenesis. Here, we have studied the hitherto neglected influence of the host substrate when reconstituting ER networks in vitro as compared to ER networks in vivo. In culture cells we observed cytoplasm-spanning ER networks with tubules being connected almost exclusively by three-way junctions and segment lengths being narrowly distributed around a mean length of about 1 μm. In contrast, networks reconstituted from purified ER microsomes on flat glass or gel substrates of varying stiffness showed significantly broader length distributions with an up to fourfold larger mean length. Self-assembly of ER microsomes on small oil droplets, however, yielded networks that resembled more closely the native ER network of mammalian cells. We conclude from these observations that the ER microsomes' inherent self-assembly capacity is sufficient to support network formation with a native geometry if the influence of the host substrate's surface chemistry becomes negligible. We hypothesize that under these conditions the networks' preference for three-way junctions follows from creating ‘starfish-shaped’ vesicles when ER microsomes with a protein-induced spontaneous curvature undergo fusion.

3.2529 Defined spatiotemporal features of RAS-ERK signals dictate cell fate in MCF-7 mammary epithelial cells

Herrero, A., Casar, B., Colon-Bolea. P., Aguda-Ibanez, L. and Crespo, P.
*Mol. Biol. Cell, **27**, 1958-1968 (2016)*

Signals conveyed through the RAS-ERK pathway are essential for the determination of cell fate. It is well established that signal variability is achieved in the different microenvironments in which signals unfold. It is also known that signal duration is critical for decisions concerning cell commitment. However, it is unclear how RAS-ERK signals integrate time and space in order to elicit a given biological response. To investigate this, we used MCF-7 cells, in which EGF-induced transient ERK activation triggers proliferation, whereas sustained ERK activation in response to heregulin leads to adipocytic differentiation.

We found that both proliferative and differentiating signals emanate exclusively from plasma membrane–disordered microdomains. Of interest, the EGF signal can be transformed into a differentiating stimulus by HRAS overexpression, which prolongs ERK activation, but only if HRAS localizes at disordered membrane. On the other hand, HRAS signals emanating from the Golgi complex induce apoptosis and can prevent heregulin-induced differentiation. Our results indicate that within the same cellular context, RAS can exert different, even antagonistic, effects, depending on its sublocalization. Thus cell destiny is defined by the ability of a stimulus to activate RAS at the appropriate sublocalization for an adequate period while avoiding switching on opposing RAS signals.

3.2530 A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles

Koeppenen, K., Hampton, T.H., jarek, M., Scharfe, M., Gerber, S.A., Mielcarz, D.W., Demers, E.G., Dolben, E.L., Hammond, J.H., Hogan, D.A. and Stanton, B.A.
*PloS Pathogens, **12(6)**, e1005672 (2016)*

Bacterial outer membrane vesicle (OMV)-mediated delivery of proteins to host cells is an important mechanism of host-pathogen communication. Emerging evidence suggests that OMVs contain differentially packaged short RNAs (sRNAs) with the potential to target host mRNA function and/or stability. In this study, we used RNA-Seq to characterize differentially packaged sRNAs in Pseudomonas aeruginosa OMVs, and to show transfer of OMV sRNAs to human airway cells. We selected one sRNA for further study based on its stable secondary structure and predicted mRNA targets. Our candidate sRNA (sRNA52320), a fragment of a P. aeruginosa methionine tRNA, was abundant in OMVs and reduced LPS-induced as well as OMV-induced IL-8 secretion by cultured primary human airway epithelial cells. We also showed that sRNA52320 attenuated OMV-induced KC cytokine secretion and neutrophil infiltration in mouse lung. Collectively, these findings are consistent with the hypothesis that sRNA52320 in OMVs is a novel mechanism of host-pathogen interaction whereby P. aeruginosa reduces the host immune response.

3.2531 Neuronal expression of ILEI/FAM3C and its reduction in Alzheimer's disease

Liu, L., Watanabe, N., Akatsu, H. and Nishimura, M.
*Neuroscience, **330**, 236-246 (2016)*

Decrease in brain amyloid-β (Aβ) accumulation is a leading strategy for treating Alzheimer's disease (AD). However, the intrinsic mechanism of the regulation of brain Aβ production is largely unknown. Previously, we reported that ILEI (also referred to as FAM3C) binds to the γ-secretase complex and suppresses Aβ production without inhibiting γ-secretase activity. In this study, we examined ILEI expression in mouse brain using immunohistochemistry and subcellular fractionation. Brain ILEI showed widespread expression in neurons and ependymal cells but not in glial and vascular endothelial cells. Neuronal ILEI resided in perinuclear vesicular structures, which were positive for a marker protein of the *trans*-Golgi network. Although ILEI immunostaining was negative at synaptic terminals, synaptosome fractionation analysis suggested that ILEI was enriched in presynaptic terminals, particularly in the active zone-docked synaptic vesicles. ILEI expression levels in brain peaked during the postnatal period and declined with age. In comparison with age-matched control brains, the number of ILEI-immunoreactive neurons decreased in AD brains, although the subcellular localization was unaltered. Our results suggest that a decline of ILEI expression may cause accumulation of Aβ in the brain and the eventual development of AD.

3.2532 Monomerization and ER Relocalization of GRASP Is a Requisite for Unconventional Secretion of CFTR

Kim, J., Noh, S.H., Piao, H., Kim, D.H., Kim, K., Cha, J.S., Chung, Y., Cho, H-S., Kim, J.Y. and Lee, M.G.
*Traffic, **17(7)**, 733-753 (2016)*

Induction of endoplasmic reticulum (ER)-to-Golgi blockade or ER stress induces Golgi reassembly stacking protein (GRASP)-mediated, Golgi-independent unconventional cell-surface trafficking of the folding-deficient ΔF508-cystic fibrosis transmembrane conductance regulator (CFTR). However, molecular mechanisms underlying this process remain elusive. Here, we show that phosphorylation-dependent dissociation of GRASP homotypic complexes and subsequent relocalization of GRASP to the ER play a critical role in the unconventional secretion of CFTR. Immunolocalization analyses of mammalian cells revealed that the Golgi protein GRASP55 was redistributed to the ER by stimuli that induce unconventional secretion of ΔF508-CFTR, such as induction of ER-to-Golgi blockade by the Arf1 mutant. Notably, the same stimuli also induced phosphorylation of regions near the C-terminus of

GRASP55 and dissociation of GRASP homomultimer complexes. Furthermore, phosphorylation-mimicking mutations of GRASP55 induced the monomerization and ER relocalization of GRASP55, and these changes were nullified by phosphorylation-inhibiting mutations. These results provide mechanistic insights into how GRASP accesses the ER-retained ΔF508-CFTR and mediates the ER stress-induced unconventional secretion pathway.

3.2533 Identification of Individual Exosome-Like Vesicles by Surface Enhanced Raman Spectroscopy
Stremersch, S., Marro, M., Pinchasik, B-El., Baatsen, P., Hendrix, A., De Smedt, S.C., Loza-Alvarez, P., Skirtach, A.G., Raemdonck, K. and Braeckmans, K.
*Small, **12(24)**, 3292-3301 (2016)*

Exosome-like vesicles (ELVs) are a novel class of biomarkers that are receiving a lot of attention for the detection of cancer at an early stage. In this study the feasibility of using a surface enhanced Raman spectroscopy (SERS) based method to distinguish between ELVs derived from different cellular origins is evaluated. A gold nanoparticle based shell is deposited on the surface of ELVs derived from cancerous and healthy cells, which enhances the Raman signal while maintaining a colloidal suspension of individual vesicles. This nanocoating allows the recording of SERS spectra from single vesicles. By using partial least squares discriminant analysis on the obtained spectra, vesicles from different origin can be distinguished, even when present in the same mixture. This proof-of-concept study paves the way for noninvasive (cancer) diagnostic tools based on exosomal SERS fingerprinting in combination with multivariate statistical analysis.

3.2534 Quantification of age-related changes of α-tocopherol in lysosomal membranes in murine tissues and human fibroblasts
König, J., Besoke, F., Stuetz, W., Malaarski, A., Jahreis, G., Grune, T. and Höhn, A.
*BioFactors, **42(3)**, 307-315 (2016)*

Considering the biological function of α-tocopherol (α-Toc) as a potent protective factor against oxidative stress, this antioxidant is in the focus of aging research. To understand the role of α-Toc during aging we investigated α-Toc concentrations in young and aged primary human fibroblasts after supplementation with RRR-α-Toc. Additionally, α-Toc contents were determined in brain, kidney, and liver tissue of 10 week-, 18 month-, and 24 month-old mice, which were fed a standard diet containing 100 mg/kg dl-α-tocopheryl acetate. α-Toc concentrations in isolated lysosomes and the expression of the α-Toc transport proteins Niemann Pick C1 (NPC1), Niemann Pick C2 (NPC2), and lipoprotein lipase were also analyzed. Obtained data show a significant age-related increase of α-Toc in murine liver, kidney, and brain tissue as well as in human dermal fibroblasts. Also liver and kidney lysosomes are marked by elevated α-Toc contents with aging. NPC1 and NPC2 protein amounts are significantly decreased in adult and aged murine kidney tissue. Also aged human dermal fibroblasts show decreased NPC1 amounts. Supplementation of young and aged fibroblasts led also to decreased NPC1 amounts, suggesting a direct role of this protein in α-Toc distribution. Our results indicate an age-dependent increase of α-Toc in different murine tissues as well as in human fibroblasts. Furthermore saturation and intracellular distribution of α-Toc seem to be strongly dependent on the availability of this vitamin as well as on the presence of the lysosomal protein NPC1.

3.2535 RNAi delivery by exosome-mimetic nanovesicles – Implications for targeting c-Myc in cancer
Lunavat, T.R., Jang, S.C., Nilsson, L., Park, H.T., Repiska, G., Lässer, C., Nilsson, J.A., Gho, Y.S. and Lötvall, J.
*Biomaterials, **102**, 231-238 (2016)*

To develop RNA-based therapeutics, it is crucial to create delivery vectors that transport the RNA molecule into the cell cytoplasm. Naturally released exosomes vesicles (also called “Extracellular Vesicles”) have been proposed as possible RNAi carriers, but their yield is relatively small in any cell culture system. We have previously generated exosome-mimetic nanovesicles (NV) by serial extrusions of cells through nano-sized filters, which results in 100-times higher yield of extracellular vesicles. We here test 1) whether NV can be loaded with siRNA exogenously and endogenously, 2) whether the siRNA-loaded NV are taken up by recipient cells, and 3) whether the siRNA can induce functional knock-down responses in recipient cells. A siRNA against GFP was first loaded into NV by electroporation, or a c-Myc shRNA was expressed inside of the cells. The NV were efficiently loaded with siRNA with both techniques, were taken up by recipient cells, which resulted in attenuation of target gene expression. In conclusion, our study suggests that exosome-mimetic nanovesicles can be a platform for RNAi delivery to

cell cytoplasm. To develop RNA-based therapeutics, it is crucial to create delivery vectors that transport the RNA molecule into the cell cytoplasm. Naturally released exosomes vesicles (also called "Extracellular Vesicles") have been proposed as possible RNAi carriers, but their yield is relatively small in any cell culture system. We have previously generated exosome-mimetic nanovesicles (NV) by serial extrusions of cells through nano-sized filters, which results in 100-times higher yield of extracellular vesicles. We here test 1) whether NV can be loaded with siRNA exogenously and endogenously, 2) whether the siRNA-loaded NV are taken up by recipient cells, and 3) whether the siRNA can induce functional knock-down responses in recipient cells. A siRNA against GFP was first loaded into NV by electroporation, or a c-Myc shRNA was expressed inside of the cells. The NV were efficiently loaded with siRNA with both techniques, were taken up by recipient cells, which resulted in attenuation of target gene expression. In conclusion, our study suggests that exosome-mimetic nanovesicles can be a platform for RNAi delivery to cell cytoplasm.

3.2536 Cathepsin Protease Controls Copper and Cisplatin Accumulation via Cleavage of the Ctr1 Metal-binding Ectodomain
Öhrvik, H., Logeman, B., Turk, B., Reinheckel, T and Thiele, D.J.
J. Biol. Chem., ***291(27)***, *13905-13916 (2016)*

Copper is an essential metal ion for embryonic development, iron acquisition, cardiac function, neuropeptide biogenesis, and other critical physiological processes. Ctr1 is a high affinity Cu^{+} transporter on the plasma membrane and endosomes that exists as a full-length protein and a truncated form of Ctr1 lacking the methionine- and histidine-rich metal-binding ectodomain, and it exhibits reduced Cu^{+} transport activity. Here, we identify the cathepsin L/B endolysosomal proteases functioning in a direct and rate-limiting step in the Ctr1 ectodomain cleavage. Cells and mice lacking cathepsin L accumulate full-length Ctr1 and hyper-accumulate copper. As Ctr1 also transports the chemotherapeutic drug cisplatin via direct binding to the ectodomain, we demonstrate that the combination of cisplatin with a cathepsin L/B inhibitor enhances cisplatin uptake and cell killing. These studies identify a new processing event and the key protease that cleaves the Ctr1 metal-binding ectodomain, which functions to regulate cellular Cu^{+} and cisplatin acquisition.

3.2537 iTRAQ-based quantitative proteomic analysis reveals the role of the tonoplast in fruit senescence
Liu, R., Wang, Y., Qin, G. and Tian, S.
J. Proteomics, ***146***, *80-89 (2016)*

The vacuole is by far the largest multifunctional organelle in fruits and plays a functional role in fruit development and fruit quality. Despite its significance, little information exists pertaining to the role of the vacuolar membrane (tonoplast) in the process of fruit senescence. In the present study, an iTRAQ-based quantitative proteomic approach was used to characterize the dynamic alterations in the tonoplast proteome during fruit senescence. Tonoplasts were purified from apple fruit at various stages of senescence using an iodixanol step gradient protocol. A total of 345 tonoplast-related proteins were identified with diverse functions such as transporters and proton pumps, signal transduction, membrane fusion or vesicle trafficking, cellular metabolic process, defense response, protein folding and degradation, and cytoskeleton. Changes in protein abundance during storage were characterized for the identified proteins. A total of 22 proteins displayed differential levels of abundance during storage. The senescence-related tonoplast proteins mostly function in the transportation of metabolites, signal transduction, membrane trafficking, and stress response. RT-qPCR analysis was used to quantify the level of expression of nine genes encoding some of the differentially abundant proteins. The results of this study provide new information regarding the function of the tonoplast during fruit senescence.

3.2538 Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges
Ha, D., Yang, N., Nadithe, V.
Acta Pharmaceutica Sinica B, ***6(4)***, *287-296 (2016)*

Exosomes are small intracellular membrane-based vesicles with different compositions that are involved in several biological and pathological processes. The exploitation of exosomes as drug delivery vehicles offers important advantages compared to other nanoparticulate drug delivery systems such as liposomes and polymeric nanoparticles; exosomes are non-immunogenic in nature due to similar composition as body's own cells. In this article, the origin and structure of exosomes as well as their biological functions are outlined. We will then focus on specific applications of exosomes as drug delivery systems in

pharmaceutical drug development. An overview of the advantages and challenges faced when using exosomes as a pharmaceutical drug delivery vehicles will also be discussed.

3.2539 Membrane Isolation Methods
Stillwell, W.
An Introduction to Biological Membranes, 247-271 (2016)

Isolating biological membrane is as much of an art form as it is a science. The procedures are tedious and require patience, precision, and organization. Since every membrane has its own peculiarities, countless procedures have been published. This chapter will consider a few of the problems encountered in isolating membranes and some of the more common techniques that have been employed.

3.2540 Membrane Reconstitution
Stillwell, W.
An Introduction to Biological Membranes, 273-312 (2016)

Membranes are unimaginably complex. They consist of hundreds of different proteins floating in a bewildering and crowded sea of a 1000 or more different lipids and this mixture is in constant flux. A membrane is not homogeneous but exists in fleeting patches that exhibit both lateral and transmembrane asymmetry. Also, countless reactions occur simultaneously in and around the membrane. Can the membrane problem be simplified? In this chapter we will examine how one membrane protein can be isolated away from the others and reconstituted into a model lipid bilayer membrane in order to determine how the protein functions. Finally, we will investigate the isolation and properties of an important membrane domain involved in cell signaling, the lipid raft.

3.2541 Cytoskeleton and Intracellular Motility
Casem, M.L.
Case Studies in Cell Biology, 127-156 (2016)

A cell's cytoskeleton provides the structural support necessary to maintain cell shape or anchor the cell to the extracellular matrix. At the same time, the cytoskeleton creates a dynamic scaffold for the movement of organelles, chromosomes, and the cell itself. The potential for integration between the different distinct cytoskeletal filament systems is investigated in the case study, "Plakins: Keeping the Cytoskeleton Safe," (Yang Y, Bauer C, Strasser G, Wollman R, Julien J-P, Fuchs E. Integrators of the cytoskeleton that stabilize microtubules. Cell 1999;98:229–238). The discovery of the microtubule motor protein kinesin (Vale RD, Schnapp BJ, Reese TS, Sheetz MP. Organelle, bead and microtubule translocations promoted by soluble factors from the squid giant axon. Cell 1985;40:559–569) sets the stage for the case study, "The *Moving* Story of a Microtubule Motor Protein." The case studies, "The WASP and the Barbed End" (Co C, Wong DT, Gierke S, Chang V, Taunton J. Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. Cell 2007;128: 901–913) and "Cilia Grow Where Vesicles Go" (Wood CR, Rosenbaum JL. Proteins of the ciliary axoneme are found on cytoplasmic membrane vesicles during growth of cilia. Curr Biol 2014; 24: 1114–1120.) investigate different aspects of the dynamic nature of the cytoskeleton.

3.2542 Role of Lipid Rafts and the Underlying Filamentous-Actin Cytoskeleton in Cannabinoid Receptor 1 Signaling
Mangoura, D., Asimaki, O., Tsirimonaki, E. and Sakellaridis, N.
*Neuropathology of Drug Addictions and Substance Misuse, **(Volume 1)**, 689-701 (2016)*

The cannabinoid 1 receptor, CB1, has evolved as a major regulatory molecule for almost all known aspects of the development and function of the central nervous system (CNS), with biological actions ranging from proper CNS cellularity to complex behaviors such as fear, appetite, and addiction. It is therefore critical to understand the mechanisms and intracellular signal transduction pathways that CB1 utilizes for its acute and long-term actions, in particular activation of the major effector extracellular signal-regulated kinase (ERK) in discrete amplification waves. This highly complex regulation of CB1 signaling to ERK is facilitated by specialized membrane microdomains, the lipid rafts. Integral components of rafts are required for proper CB1 presentation at the plasma membrane as shown by confocal analysis, while the dynamic and hierarchic activation of its major proximal effectors protein kinase Cε, Src, and Fyn requires raft integrity, additionally causing fibroblast growth factor receptor transactivation. Thus, lipid rafts constitute the plasma membrane platform on which CB1 signaling is initiated and organized.

3.2543 **Septin 9 induces lipid droplets growth by a phosphatidylinositol-5-phosphate and microtubule-dependent mechanism hijacked by HCV**
Akil, A. et al
*Nature Communications, **7**:12203 (2016)*

The accumulation of lipid droplets (LD) is frequently observed in hepatitis C virus (HCV) infection and represents an important risk factor for the development of liver steatosis and cirrhosis. The mechanisms of LD biogenesis and growth remain open questions. Here, transcriptome analysis reveals a significant upregulation of septin 9 in HCV-induced cirrhosis compared with the normal liver. HCV infection increases septin 9 expression and induces its assembly into filaments. Septin 9 regulates LD growth and perinuclear accumulation in a manner dependent on dynamic microtubules. The effects of septin 9 on LDs are also dependent on binding to PtdIns5P, which, in turn, controls the formation of septin 9 filaments and its interaction with microtubules. This previously undescribed cooperation between PtdIns5P and septin 9 regulates oleate-induced accumulation of LDs. Overall, our data offer a novel route for LD growth through the involvement of a septin 9/PtdIns5P signalling pathway.

3.2544 **AP-1/σ1A and AP-1/σ1B adaptor-proteins differentially regulate neuronal early endosome maturation via the Rab5/Vps34-pathway**
Candiello, E., Kratzke, M., Wenzel, d., Cassel, D. and Schu, P.
*Scientific Reports, **6**:29950 (2015)*

The σ1 subunit of the AP-1 clathrin-coated-vesicle adaptor-protein complex is expressed as three isoforms. Tissues express σ1A and one of the σ1B and σ1C isoforms. Brain is the tissue with the highest σ1A and σ1B expression. σ1B-deficiency leads to severe mental retardation, accumulation of early endosomes in synapses and fewer synaptic vesicles, whose recycling is slowed down. AP-1/σ1A and AP-1/σ1B regulate maturation of these early endosomes into multivesicular body late endosomes, thereby controlling synaptic vesicle protein transport into a degradative pathway. σ1A binds ArfGAP1, and with higher affinity brain-specific ArfGAP1, which bind Rabex-5. AP-1/σ1A-ArfGAP1-Rabex-5 complex formation leads to more endosomal Rabex-5 and enhanced, Rab5GTP-stimulated Vps34 PI3-kinase activity, which is essential for multivesicular body endosome formation. Formation of AP-1/σ1A-ArfGAP1-Rabex-5 complexes is prevented by σ1B binding of Rabex-5 and the amount of endosomal Rabex-5 is reduced. AP-1 complexes differentially regulate endosome maturation and coordinate protein recycling and degradation, revealing a novel molecular mechanism by which they regulate protein transport besides their established function in clathrin-coated-vesicle formation.

3.2545 **Limited ER quality control for GPI-anchored proteins**
Sikorska, N., Lemus, L., Aguilera-Romero, A., Monzano-Lopez, J., Riezman, H., Muniz, M. and Goder, V.
*J. Cell Biol., **213(6)**, 693-704 (2016)*

Endoplasmic reticulum (ER) quality control mechanisms target terminally misfolded proteins for ER-associated degradation (ERAD). Misfolded glycophosphatidylinositol-anchored proteins (GPI-APs) are, however, generally poor ERAD substrates and are targeted mainly to the vacuole/lysosome for degradation, leading to predictions that a GPI anchor sterically obstructs ERAD. Here we analyzed the degradation of the misfolded GPI-AP Gas1* in yeast. We could efficiently route Gas1* to Hrd1-dependent ERAD and provide evidence that it contains a GPI anchor, ruling out that a GPI anchor obstructs ERAD. Instead, we show that the normally decreased susceptibility of Gas1* to ERAD is caused by canonical remodeling of its GPI anchor, which occurs in all GPI-APs and provides a protein-independent ER export signal. Thus, GPI anchor remodeling is independent of protein folding and leads to efficient ER export of even misfolded species. Our data imply that ER quality control is limited for the entire class of GPI-APs, many of them being clinically relevant.

3.2546 **Cortactin promotes exosome secretion by controlling branched actin dynamics**
Sinha, S., Hoshino, D., Hong, N.H., Kirkbride, K.C., Grega-larson, E., Seiki, M., Tyska, M.J. and Weaver, A.M.
*J. Cell Biol., **214(2)**, 197-213 (2016)*

Exosomes are extracellular vesicles that influence cellular behavior and enhance cancer aggressiveness by carrying bioactive molecules. The mechanisms that regulate exosome secretion are poorly understood.

Here, we show that the actin cytoskeletal regulatory protein cortactin promotes exosome secretion. Knockdown or overexpression of cortactin in cancer cells leads to a respective decrease or increase in exosome secretion, without altering exosome cargo content. Live-cell imaging revealed that cortactin controls both trafficking and plasma membrane docking of multivesicular late endosomes (MVEs). Regulation of exosome secretion by cortactin requires binding to the branched actin nucleating Arp2/3 complex and to actin filaments. Furthermore, cortactin, Rab27a, and coronin 1b coordinately control stability of cortical actin MVE docking sites and exosome secretion. Functionally, the addition of purified exosomes to cortactin-knockdown cells rescued defects of those cells in serum-independent growth and invasion. These data suggest a model in which cortactin promotes exosome secretion by stabilizing cortical actin-rich MVE docking sites.

3.2547 Effect of Rhodopsin Phosphorylation on Dark Adaptation in Mouse Rods
Berry, J., Frederiksen, R., Yao, Y., Nymark, S., Chen, J. and Cornwall, C.
J. Neurosci., ***36(26)****, 6973-6987 (2016)*

Rhodopsin is a prototypical G-protein-coupled receptor (GPCR) that is activated when its 11-*cis*-retinal moiety is photoisomerized to all-*trans* retinal. This step initiates a cascade of reactions by which rods signal changes in light intensity. Like other GPCRs, rhodopsin is deactivated through receptor phosphorylation and arrestin binding. Full recovery of receptor sensitivity is then achieved when rhodopsin is regenerated through a series of steps that return the receptor to its ground state. Here, we show that dephosphorylation of the opsin moiety of rhodopsin is an extremely slow but requisite step in the restoration of the visual pigment to its ground state. We make use of a novel observation: isolated mouse retinae kept in standard media for routine physiologic recordings display blunted dephosphorylation of rhodopsin. Isoelectric focusing followed by Western blot analysis of bleached isolated retinae showed little dephosphorylation of rhodopsin for up to 4 h in darkness, even under conditions when rhodopsin was completely regenerated. Microspectrophotometeric determinations of rhodopsin spectra show that regenerated phospho-rhodopsin has the same molecular photosensitivity as unphosphorylated rhodopsin and that flash responses measured by *trans*-retinal electroretinogram or single-cell suction electrode recording displayed dark-adapted kinetics. Single quantal responses displayed normal dark-adapted kinetics, but rods were only half as sensitive as those containing exclusively unphosphorylated rhodopsin. We propose a model in which light-exposed retinae contain a mixed population of phosphorylated and unphosphorylated rhodopsin. Moreover, complete dark adaptation can only occur when all rhodopsin has been dephosphorylated, a process that requires >3 h in complete darkness.

3.2548 Immunization with Outer Membrane Vesicles Displaying Designer Glycotopes Yields Class-Switched, Glycan-Specific Antibodies
Valentine, J.L., Chen, L., Perregaux, E.C. et al
Cell Chem. Biol., ***23(6)****, 655-665 (2016)*

The development of antibodies against specific glycan epitopes poses a significant challenge due to difficulties obtaining desired glycans at sufficient quantity and purity, and the fact that glycans are usually weakly immunogenic. To address this challenge, we leveraged the potent immunostimulatory activity of bacterial outer membrane vesicles (OMVs) to deliver designer glycan epitopes to the immune system. This approach involved heterologous expression of two clinically important glycans, namely polysialic acid (PSA) and Thomsen-Friedenreich antigen (T antigen) in hypervesiculating strains of non-pathogenic *Escherichia coli.* The resulting glycOMVs displayed structural mimics of PSA or T antigen on their surfaces, and induced high titers of glycan-specific IgG antibodies following immunization in mice. In the case of PSA glycOMVs, serum antibodies potently killed *Neisseria meningitidis* serogroup B (MenB), whose outer capsule is PSA, in a serum bactericidal assay. These findings demonstrate the potential of glycOMVs for inducing class-switched, humoral immune responses against glycan antigens.

3.2549 Outer membrane vesicles displaying engineered glycotopes elicit protective antibodies
Chen, L. et al
PNAS, ***113(26)****, E3609-E3618 (2016)*

The O-antigen polysaccharide (O-PS) component of lipopolysaccharides on the surface of gram-negative bacteria is both a virulence factor and a B-cell antigen. Antibodies elicited by O-PS often confer protection against infection; therefore, O-PS glycoconjugate vaccines have proven useful against a number of different pathogenic bacteria. However, conventional methods for natural extraction or chemical synthesis of O-PS are technically demanding, inefficient, and expensive. Here, we describe an alternative

methodology for producing glycoconjugate vaccines whereby recombinant O-PS biosynthesis is coordinated with vesiculation in laboratory strains of *Escherichia coli* to yield glycosylated outer membrane vesicles (glycOMVs) decorated with pathogen-mimetic glycotopes. Using this approach, glycOMVs corresponding to eight different pathogenic bacteria were generated. For example, expression of a 17-kb O-PS gene cluster from the highly virulent *Francisella tularensis* subsp. *tularensis* (type A) strain Schu S4 in hypervesiculating *E. coli* cells yielded glycOMVs that displayed *F. tularensis* O-PS. Immunization of BALB/c mice with glycOMVs elicited significant titers of O-PS–specific serum IgG antibodies as well as vaginal and bronchoalveolar IgA antibodies. Importantly, glycOMVs significantly prolonged survival upon subsequent challenge with *F. tularensis* Schu S4 and provided complete protection against challenge with two different *F. tularensis* subsp. *holarctica* (type B) live vaccine strains, thereby demonstrating the vaccine potential of glycOMVs. Given the ease with which recombinant glycotopes can be expressed on OMVs, the strategy described here could be readily adapted for developing vaccines against many other bacterial pathogens.

3.2550 ATP: The crucial component of secretory vesicles
Estevez-herrera, J., Dominguez, N., pardo, M.R., Gonzalez-Santana, A., Westhead, E.W., Borges, R. and Machado, J.D.
*PNAS, **113(28)**, E4098-E4106 (2016)*

The colligative properties of ATP and catecholamines demonstrated in vitro are thought to be responsible for the extraordinary accumulation of solutes inside chromaffin cell secretory vesicles, although this has yet to be demonstrated in living cells. Because functional cells cannot be deprived of ATP, we have knocked down the expression of the vesicular nucleotide carrier, the VNUT, to show that a reduction in vesicular ATP is accompanied by a drastic fall in the quantal release of catecholamines. This phenomenon is particularly evident in newly synthesized vesicles, which we show are the first to be released. Surprisingly, we find that inhibiting VNUT expression also reduces the frequency of exocytosis, whereas the overexpression of VNUT drastically increases the quantal size of exocytotic events. To our knowledge, our data provide the first demonstration that ATP, in addition to serving as an energy source and purinergic transmitter, is an essential element in the concentration of catecholamines in secretory vesicles. In this way, cells can use ATP to accumulate neurotransmitters and other secreted substances at high concentrations, supporting quantal transmission.

3.2551 Reversible HuR-microRNA binding controls extracellular export of miR-122 and augments stress response
Mukherjee, K., Ghoshai, B., Ghosh, S., Chakrabarty, Y., Shwetha, S., Das, S. and Bhattacharyya, S.N.
*EMBO Reports, **17(8)**, 1184-1203 (2016)*

microRNAs (miRNAs), the tiny but stable regulatory RNAs in metazoan cells, can undergo selective turnover in presence of specific internal and external cues to control cellular response against the changing environment. We have observed reduction in cellular miR-122 content, due to their accelerated extracellular export in human hepatic cells starved for small metabolites including amino acids. In this context, a new role of human ELAV protein HuR has been identified. HuR, a negative regulator of miRNA function, accelerates extracellular vesicle (EV)-mediated export of miRNAs in human cells. In stressed cells, HuR replaces miRNPs from target messages and is both necessary and sufficient for the extracellular export of corresponding miRNAs. HuR could reversibly bind miRNAs to replace them from Ago2 and subsequently itself gets freed from bound miRNAs upon ubiquitination. The ubiquitinated form of HuR is predominantly associated with multivesicular bodies (MVB) where HuR-unbound miRNAs also reside. These MVB-associated pool of miRNAs get exported out via EVs thereby delimiting cellular miR-122 level during starvation. Therefore, by modulating extracellular export of miR-122, HuR could control stress response in starved human hepatic cells.

3.2552 Spatial control of lipid droplet proteins by the ERAD ubiquitin ligase Doa10
Ruggiano, A., Mora, G., Buxo, L. and Carvalho, P.
*EMBO J., **35(15)**, 1644-1655 (2016)*

The endoplasmic reticulum (ER) plays a central role in the biogenesis of most membrane proteins. Among these are proteins localized to the surface of lipid droplets (LDs), fat storage organelles delimited by a phospholipid monolayer. The LD monolayer is often continuous with the membrane of the ER allowing certain membrane proteins to diffuse between the two organelles. In these connected organelles, how some proteins concentrate specifically at the surface of LDs is not known. Here, we show that the ERAD

ubiquitin ligase Doa10 controls the levels of some LD proteins. Their degradation is dependent on the localization to the ER and appears independent of the folding state. Moreover, we show that by degrading the ER pool of these LD proteins, ERAD contributes to restrict their localization to LDs. The signals for LD targeting and Doa10-mediated degradation overlap, indicating that these are competing events. This spatial control of protein localization is a novel function of ERAD that might contribute to generate functional diversity in a continuous membrane system.

3.2553 Brefeldin A promotes the appearance of oligosaccharyl phosphates derived from Glc3Man9GlcNAc2-PP-dolichol within the endomembrane system of HepG2 cells
Massarweh, A., Bosco, M., Iatmanen-harbi, S., Tessier, C., Amana, L., Busca, P., Chantret, I., Gravier-Pelletier, C. and Moore, S.E.H.
*J. Lipid Res., **57**, 1477-1491 (2016)*

We reported an oligosaccharide diphosphodolichol (DLO) diphosphatase (DLODP) that generates dolichyl-phosphate and oligosaccharyl phosphates (OSPs) from DLO in vitro. This enzyme could underlie cytoplasmic OSP generation and promote dolichyl-phosphate recycling from truncated endoplasmic reticulum (ER)-generated DLO intermediates. However, during subcellular fractionation, DLODP distribution is closer to that of a Golgi apparatus (GA) marker than those of ER markers. Here, we examined the effect of brefeldin A (BFA), which fuses the GA with the ER on OSP metabolism. In order to increase the steady state level of truncated DLO while allowing formation of mature DLO ($Glc_3Man_9GlcNAc_2$-PP-dolichol), dolichyl-P-mannose $Man_7GlcNAc_2$-PP-dolichol mannosyltransferase was partially downregulated in HepG2 cells. We show that BFA provokes GA endomannosidase trimming of $Glc_3Man_9GlcNAc_2$-PP-dolichol to yield a $Man_8GlcNAc_2$-PP-dolichol structure that does not give rise to cytoplasmic $Man_8GlcNAc_2$-P. BFA also strikingly increased OSP derived from mature DLO within the endomembrane system without affecting levels of $Man_7GlcNAc_2$-PP-dolichol or cytoplasmic $Man_7GlcNAc_2$-P. The BFA-provoked increase in endomembrane-situated OSP is sensitive to nocodazole, and BFA causes partial redistribution of DLODP activity from GA- to ER-containing regions of density gradients. These findings are consistent with BFA-provoked microtubule-dependent GA-to-ER transport of a previously reported DLODP that acts to generate a novel endomembrane-situated OSP population.

3.2554 Caspase-cleaved Tau-D421 is colocalized with the immunophilin FKBP52 in the autophagy-endolysosomal system of Alzheimer's disease neurons
Meduri, G., Guillemeau, K., Dounane, O., Sazdovitch, V., Duyckaerts, C., Chambraud, B., Baulieu, E.E. and Giustiniani, J.
*Neurobiology of Aging, **46**, 124-137 (2016)*

Pathologic modifications of the Tau protein leading to neurofibrillary tangle (NFT) formation are a common feature of a wide range of neurodegenerative diseases known as tauopathies, which include Alzheimer's disease (AD). We previously showed that the immunophilin FKBP52 physically and functionally interacts with Tau, and we recently reported that FKBP52 levels are abnormally low in AD patients' brains. To decipher the mechanism of FKBP52 decrease in AD brains, we performed multiple labeling immunohistofluorescence and lysosomal purification using postmortem brain samples of healthy controls (n = 8) and AD (n = 20) patients. Confocal analysis revealed that FKBP52 localizes to the endolysosomal system. We also report FKBP52 colocalization with the truncated Tau-D^{421} in the autophagy-endolysosomal system in some AD neurons and that the decrease of FKBP52 correlates with NFT formation. Additional experiments of autophagy inhibition in Tau-inducible SH-SY5Y cells allowed demonstrating FKBP52 release in the extracellular milieu. Our findings point out the possibility that FKBP52 could be abnormally released from NFTs negative neurons in AD brains in correlation with the early pathologic Tau-D^{421} neuronal accumulation.

3.2555 Translocation of the ABC transporter ABCD4 from the endoplasmic reticulum to lysosomes requires the escort protein LMBD1
Kawaguchi, K., Okamoto, T., Morita, M. and Imanaka, T.
*Scientific Reports, **6**:30183 (2016)*

We previously demonstrated that ABCD4 does not localize to peroxisomes but rather, the endoplasmic reticulum (ER), because it lacks the NH2-terminal hydrophilic region required for peroxisomal targeting. It was recently reported that mutations in ABCD4 result in a failure to release vitamin B12 from lysosomes. A similar phenotype is caused by mutations in LMBRD1, which encodes the lysosomal membrane protein LMBD1. These findings suggested to us that ABCD4 translocated from the ER to lysosomes in association

with LMBD1. In this report, it is demonstrated that ABCD4 interacts with LMBD1 and then localizes to lysosomes, and this translocation depends on the lysosomal targeting ability of LMBD1. Furthermore, endogenous ABCD4 was localized to both lysosomes and the ER, and its lysosomal localization was disturbed by knockout of LMBRD1. To the best of our knowledge, this is the first report demonstrating that the subcellular localization of the ABC transporter is determined by its association with an adaptor protein.

3.2556 Acetylation modification regulates GRP78 secretion in colon cancer cells
Li, Z., Zhuang, M., Zhang, L., Zheng, X., Yang, P. and Li, Z.
*Scientific Reports, **6**:30406 (2016)*

High glucose-regulated protein 78 (GRP78) expression contributes to the acquisition of a wide range of phenotypic cancer hallmarks, and the pleiotropic oncogenic functions of GRP78 may result from its diverse subcellular distribution. Interestingly, GRP78 has been reported to be secreted from solid tumour cells, participating in cell-cell communication in the tumour microenvironment. However, the mechanism underlying this secretion remains elusive. Here, we report that GRP78 is secreted from colon cancer cells via exosomes. Histone deacetylase (HDAC) inhibitors blocked GRP78 release by inducing its aggregation in the ER. Mechanistically, HDAC inhibitor treatment suppressed HDAC6 activity and led to increased GRP78 acetylation; acetylated GRP78 then bound to VPS34, a class III phosphoinositide-3 kinase, consequently preventing the sorting of GRP78 into multivesicular bodies (MVBs). Of note, we found that mimicking GRP78 acetylation by substituting the lysine at residue 633, one of the deacetylated sites of HDAC6, with a glutamine resulted in decreased GRP78 secretion and impaired tumour cell growth in vitro. Our study thus reveals a hitherto-unknown mechanism of GRP78 secretion and may also provide implications for the therapeutic use of HDAC inhibitors.

3.2557 Legionella pneumophila Type IV Effectors YlfA and YlfB Are SNARE-Like Proteins that Form Homo- and Heteromeric Complexes and Enhance the Efficiency of Vacuole Remodeling
Campodonico, E.M., Roy, C.R. and Ninio, S.
*Plos One, **11(7)**, e0159698 (2016)*

Legionella pneumophila is a Gram-negative bacterium that can colonize both freshwater protozoa and human alveolar macrophages, the latter infection resulting in Legionnaires' disease. The intracellular lifecycle of L. pneumophila requires extensive manipulation of its host cell, which is carried out by effector proteins that are translocated into the host cell through the Dot/Icm type IV secretion system. This study focuses on a pair of highly similar type IV substrates called YlfA/LegC7 and YlfB/LegC2 that were initially identified in a screen for proteins that cause growth inhibition in yeast. Analysis of truncation mutants revealed that the hydrophobic residues in the Ylf amino termini were required for localization of each protein to the membranes of host cells. Central and carboxy terminal coiled coil domains were found to mediate binding of YlfA and YlfB to themselves and to each other. In vivo, a ΔylfA ΔylfB double mutant strain of L. pneumophila was shown to be defective in establishing a vacuole that supports bacterial replication. This phenotype was subsequently correlated with a decrease in the association of endoplasmic reticulum (ER)-derived vesicles with vacuoles containing ΔylfA ΔylfB mutant bacteria. These data suggest that the Ylf proteins are membrane-associated effectors that enhance remodeling of the L. pneumophila -containing vacuole by promoting association and possibly fusion of ER-derived membrane vesicles with the bacterial compartment.

3.2558 Uropathogenic Escherichia coli Releases Extracellular Vesicles That Are Associated with RNA
Blenkiron, C., Simonov, D., Muthukaruppan, A., Tsai, P., Dauros, P., Green, S., hong, J., Print, C.G., Swift, S. and Phillips, A.
*PloS One, **11(8)**, e0160440 (2016)*

Background
Bacterium-to-host signalling during infection is a complex process involving proteins, lipids and other diffusible signals that manipulate host cell biology for pathogen survival. Bacteria also release membrane vesicles (MV) that can carry a cargo of effector molecules directly into host cells. Supported by recent publications, we hypothesised that these MVs also associate with RNA, which may be directly involved in the modulation of the host response to infection.
Methods and Results
Using the uropathogenic Escherichia coli (UPEC) strain 536, we have isolated MVs and found they carry a range of RNA species. Density gradient centrifugation further fractionated and characterised the MV

preparation and confirmed that the isolated RNA was associated with the highest particle and protein containing fractions. Using a new approach, RNA-sequencing of libraries derived from three different 'size' RNA populations (<50nt, 50-200nt and 200nt+) isolated from MVs has enabled us to now report the first example of a complete bacterial MV-RNA profile. These data show that MVs carry rRNA, tRNAs, other small RNAs as well as full-length protein coding mRNAs. Confocal microscopy visualised the delivery of lipid labelled MVs into cultured bladder epithelial cells and showed their RNA cargo labelled with 5-EU (5-ethynyl uridine), was transported into the host cell cytoplasm and nucleus. MV RNA uptake by the cells was confirmed by droplet digital RT-PCR of csrC. It was estimated that 1% of MV RNA cargo is delivered into cultured cells.
Conclusions
These data add to the growing evidence of pathogenic bacterial MV being associated a wide range of RNAs. It further raises the plausibility for MV-RNA-mediated cross-kingdom communication whereby they influence host cell function during the infection process.

3.2559 181 VESICLE-ASSOCIATED MEMBRANE PROTEIN 7 IS CRUCIAL FOR LIPID ABSORPTION

Mansbach, C.M., Siddiqi, S. and Mahan, J.
J. Investig. Med., ***54****, S288 (2016)*

The rate-limiting step in lipid absorption is the exit of triacylglycerols (TAG) from the endoplasmic reticulum (ER) in a specialized transport vesicle, the prechylomicron transport vesicle (PCTV). SNARE proteins direct vesicles to target membranes. Newly synthesized proteins in vesicles constantly move to the cis Golgi. We questioned if intermittent, meal-derived PCTV contained a unique SNARE. Vesicle-associated membrane protein 7 (VAMP7), a SNARE protein previously found only in the post Golgi compartment, was identified by 2D gels in PCTV. We sought its presence in its parent, intestinal ER, and questioned its functionality in lipid transport.
Methods We used our novel antirat VAMP7 antibody that identifies a 25 kDa protein on a 2D gel immunoblot (IB) to identify VAMP7. We isolated ER both by sucrose density and iodixanol gradients. Immunohistochemistry (IH) resolved by deconvolution was used to colocalize marker proteins with VAMP7 and immunoelectron microscopy (IEM) was used to colocalize VAMP7 to ER marker proteins on PCTV.
Results Our intestinal, liver and kidney ER preparations were not contaminated by endosomes or Golgi as judged by the lack of rab11, syntaxin8, and GOS28 in the area of the gradient occupied by the ER proteins calreticulin and sec22. Intestinal ER but not liver or kidney ER contained VAMP7 by IB. ER-derived PCTV contained VAMP7 and the ER proteins Sar1 and rBet1 by IEM. IH showed VAMP7 to be colocalized with the ER protein PDI. To test the functionality of VAMP7 in TAG transport to the Golgi, we incubated intestinal ER with anti-VAMP7 antibody and observed its effect on delivery of ER-^{14} C-TAG and protein to the Golgi. Only 10% of the ^{14} C-TAG was transported to the Golgi on anti-VAMP7 antibody treatment as compared to preincubation of the ER with IgG. Newly synthesized ^{3} H-protein transport was unaffected by anti-VAMP7 antibody as was ER to Golgi TAG transport if the Golgi was preincubated with the antibody. In coimmunoprecipitation studies, VAMP7 was associated with apolipoprotein B48 (apoB48). VAMP7 does not bind to apoB48 in intestinal ER treated with proteinase K, which left a 170 kDa apoB48 fragment.
Conclusions We have localized VAMP7 to intestinal ER by IH and IEM and by cell organelle separation techniques and shown that intestine but not liver or kidney ER contains VAMP7. We have also shown that VAMP7 in intestinal ER is functional in the movement of TAG in PCTV from ER to the Golgi. We speculate that VAMP7 binds to a cytosolic exposed portion of apoB48, which enables selection of the prechylomicron for inclusion in PCTV.

3.2560 Mannosidase IA is in Quality Control Vesicles and Participates in Glycoprotein Targeting to ERAD

Ogen-Shtern, N., Avezov, E., Shenkman, M., Benyair, R. and Lederkremer, G.Z.
J. Mol. Biol., ***428****, 3194-3205 (2016)*

Endoplasmic reticulum-associated degradation (ERAD) of a misfolded glycoprotein in mammalian cells requires the removal of 3–4 alpha 1,2 linked mannose residues from its *N*-glycans. The trimming and recognition processes are ascribed to ER Mannosidase I, the ER-degradation enhancing mannosidase-like proteins (EDEMs), and the lectins OS-9 and XTP3-B, all residing in the ER, the ER-derived quality control compartment (ERQC), or quality control vesicles (QCVs). Folded glycoproteins with untrimmed glycans are transported from the ER to the Golgi complex, where they are substrates of other alpha 1,2 mannosidases, IA, IB, and IC. The apparent redundancy of these enzymes has been puzzling for many

years. We have now determined that, surprisingly, mannosidase IA is not located in the Golgi but resides in QCVs. We had recently described this type of vesicles, which carry ER α1,2 mannosidase I (ERManI). We show that the overexpression of alpha class I α1,2 mannosidase IA (ManIA) significantly enhances the degradation of ERAD substrates and its knockdown stabilizes it. Our results indicate that ManIA trims mannose residues from $Man_9GlcNAc_2$ down to $Man_5GlcNAc_2$, acting in parallel with ERManI and the EDEMs, and targeting misfolded glycoproteins to ERAD.

3.2561 Isolation of human salivary extracellular vesicles by iodixanol density gradient ultracentrifugation and their characterizations

Iwai, K., Minamisawa, T., Suga, K., Yajima, Y. and Shiba, K.
*J. Extracellular Vesicles, **5**:30829 (2016)*

Diagnostic methods that focus on the extracellular vesicles (EVs) present in saliva have been attracting great attention because of their non-invasiveness. EVs contain biomolecules such as proteins, messenger RNA (mRNA) and microRNA (miRNA), which originate from cells that release EVs, making them an ideal source for liquid biopsy. Although there have been many reports on density-based fractionation of EVs from blood and urine, the number of reports on EVs from saliva has been limited, most probably because of the difficulties in separating EVs from viscous saliva using density gradient centrifugation. This article establishes a protocol for the isolation of EVs from human saliva using density gradient centrifugation. The fractionated salivary EVs were characterized by atomic force microscopy, western blot and reverse transcription polymerase chain reaction. The results indicate that salivary EVs have a smaller diameter (47.8±12.3 nm) and higher density (1.11 g/ml) than EVs isolated from conditioned cell media (74.0±23.5 nm and 1.06 g/ml, respectively). Additionally, to improve the throughput of density-based fractionation of EVs, the original protocol was further modified by using a fixed angle rotor instead of a swinging rotor. It was also confirmed that several miRNAs were expressed strongly in the EV-marker-expressing fractions.

3.2562 Exosomal miRNAs as cancer biomarkers and therapeutic targets

Thind, A. and Wilson, C.
*J. Extracellular Vesicles, **5**:31292 (2016)*

Intercommunication between cancer cells and with their surrounding and distant environments is key to the survival, progression and metastasis of the tumour. Exosomes play a role in this communication process. MicroRNA (miRNA) expression is frequently dysregulated in tumour cells and can be reflected by distinct exosomal miRNA (ex-miRNA) profiles isolated from the bodily fluids of cancer patients. Here, the potential of ex-miRNA as a cancer biomarker and therapeutic target is critically analysed. Exosomes are a stable source of miRNA in bodily fluids but, despite a number of methods for exosome extraction and miRNA quantification, their suitability for diagnostics in a clinical setting is questionable. Furthermore, exosomally transferred miRNAs can alter the behaviour of recipient tumour and stromal cells to promote oncogenesis, highlighting a role in cell communication in cancer. However, our incomplete understanding of exosome biogenesis and miRNA loading mechanisms means that strategies to target exosomes or their transferred miRNAs are limited and not specific to tumour cells. Therefore, if ex-miRNA is to be employed in novel non-invasive diagnostic approaches and as a therapeutic target in cancer, two further advances are necessary: in methods to isolate and detect ex-miRNA, and a better understanding of their biogenesis and functions in tumour-cell communication.

3.2563 Nanoparticle analysis sheds budding insights into genetic drivers of extracellular vesicle biogenesis

Hurwitz, S.N., Conlon, M.M., Rider, M.A., Brownstein, N.C. and Meckes Jr., D.G.
*J. Extracellular Vesicles, **5**:31295 (2016)*

Background: Extracellular vesicles (EVs) are important mediators of cell-to-cell communication in healthy and pathological environments. Because EVs are present in a variety of biological fluids and contain molecular signatures of their cell or tissue of origin, they have great diagnostic and prognostic value. The ability of EVs to deliver biologically active proteins, RNAs and lipids to cells has generated interest in developing novel therapeutics. Despite their potential medical use, many of the mechanisms underlying EV biogenesis and secretion remain unknown.
Methods: Here, we characterized vesicle secretion across the NCI-60 panel of human cancer cells by nanoparticle tracking analysis. Using CellMiner, the quantity of EVs secreted by each cell line was compared to reference transcriptomics data to identify gene products associated with vesicle secretion.

Results: Gene products positively associated with the quantity of exosomal-sized vesicles included vesicular trafficking classes of proteins with Rab GTPase function and sphingolipid metabolism. Positive correlates of larger microvesicle-sized vesicle secretion included gene products involved in cytoskeletal dynamics and exocytosis, as well as Rab GTPase activation. One of the identified targets, CD63, was further evaluated for its role in vesicle secretion. Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 knockout of the CD63 gene in HEK293 cells resulted in a decrease in small vesicle secretion, suggesting the importance of CD63 in exosome biogenesis.
Conclusion: These observations reveal new insights into genes involved in exosome and microvesicle formation, and may provide a means to distinguish EV sub-populations. This study offers a foundation for further exploration of targets involved in EV biogenesis and secretion.

3.2564 Synovial fluid pretreatment with hyaluronidase facilitates isolation of CD44+ extracellular vesicles
Boere, J., van de Lest, C.H.A., Libregts, S.F.W.M., Arkesteijn, G.J.A., Geerts, W.J.C., Nolte-t' Hoen, E.N.M., malda, J., van Weeren, P.R. and Wauben, M.H.M.
*J. Extracellular Vesicles, **5**:31751 (2016)*

Extracellular vesicles (EVs) in synovial fluid (SF) are gaining increased recognition as important factors in joint homeostasis, joint regeneration, and as biomarkers of joint disease. A limited number of studies have investigated EVs in SF samples of patients with joint disease, but knowledge on the role of EVs in healthy joints is lacking. In addition, no standardized protocol is available for isolation of EVs from SF. Based on the high viscosity of SF caused by high concentrations of hyaluronic acid (HA) – a prominent extracellular matrix component – it was hypothesized that EV recovery could be optimized by pretreatment with hyaluronidase (HYase). Therefore, the efficiency of EV isolation from healthy equine SF samples was tested by performing sequential ultracentrifugation steps (10,000*g*, 100,000*g* and 200,000*g*) in the presence or absence of HYase. Quantitative EV analysis using high-resolution flow cytometry showed an efficient recovery of EVs after 100,000*g* ultracentrifugation, with an increased yield of CD44+ EVs when SF samples were pretreated with HYase. Morphological analysis of SF-derived EVs with cryo-transmission-electron microscopy did not indicate damage by high-speed ultracentrifugation and revealed that most EVs are spherical with a diameter of 20–200 nm. Further protein characterization by Western blotting revealed that healthy SF-derived EVs contain CD9, Annexin-1, and CD90/Thy1.1. Taken together, these data suggest that EV isolation protocols for body fluids that contain relatively high amounts of HA, such as SF, could benefit from treatment of the fluid with HYase prior to ultracentrifugation. This method facilitates recovery and detection of CD44+ EVs within the HA-rich extracellular matrix. Furthermore, based on the findings presented here, it is recommended to sediment SF-derived EVs with at least 100,000*g* for optimal EV recovery.

3.2565 Regional Regulation of Purkinje Cell Dendritic Spines by Integrins and Eph/Ephrins
Heintz, T.G., Eva, R. And Fawcett, J.W.
PloS One, 11(8), e0158558 (2016)

Climbing fibres and parallel fibres compete for dendritic space on Purkinje cells in the cerebellum. Normally, climbing fibres populate the proximal dendrites, where they suppress the multiple small spines typical of parallel fibres, leading to their replacement by the few large spines that contact climbing fibres. Previous work has shown that ephrins acting via EphA4 are a signal for this change in spine type and density. We have used an in vitro culture model in which to investigate the ephrin effect on Purkinje cell dendritic spines and the role of integrins in these changes. We found that integrins α3, α5 and β4 are present in many of the dendritic spines of cultured Purkinje cells. pFAK, the main downstream signalling molecule from integrins, has a similar distribution, although the intenstity of pFAK staining and the percentage of pFAK+ spines was consistently higher in the proximal dendrites. Activating integrins with Mg2+ led to an increase in the intensity of pFAK staining and an increase in the proportion of pFAK+ spines in both the proximal and distal dendrites, but no change in spine length, density or morphology. Blocking integrin binding with an RGD-containing peptide led to a reduction in spine length, with more stubby spines on both proximal and distal dendrites. Treatment of the cultures with ephrinA3-Fc chimera suppressed dendritic spines specifically on the proximal dendrites and there was also a decrease of pFAK in spines on this domain. This effect was blocked by simultaneous activation of integrins with Mn2+. We conclude that Eph/ephrin signaling regulates proximal dendritic spines in Purkinje cells by inactivating integrin downstream signalling.

3.2566 Abstract LB-266: Large oncosomes reprogram prostate fibroblasts toward a pro-angiogenic phenotype

Minciacchi, V., Spinelli, C., Reis-Sobreiro, M., Zandian, M., Adam, R.M., Posadas, E.M., Micheal, F.R., Cocucci, E., Bhowmich, N.and Di Vizio, D.
Cancer Res., ***76(14)***, *LB-266 (2016)*

Introduction: Cancer cells communicate with different cells in the tumor microenvironment, establishing a supportive stroma that sustains tumor development and facilitates the first steps of metastasis. Extracellular vesicles (EVs) have emerged as key functional mediators of this process. Aim of this study was to determine the mechanism of intercellular communication mediated by the atypically large EVs produced by highly migratory and metastatic tumor cells, referred to as large oncosomes (LO), and prostate fibroblasts (NAF). Methods: Filtration, differential centrifugation followed by iodixanol gradient; flow cytometry and confocal imaging; RNA-seq; kinase assay; TF array; luciferase assay; tube formation; siRNA; RT-qPCR. Results: Active AKT1 is significantly more expressed and functional in LO than in exosomes (Exo). Patients with metastatic disease express abundant active AKT1 in plasma LO. Uptake of LO harboring active AKT1 by NAF results in AKT1 and c-MYC activation. Conditioned media from LO-treated NAF, but not from Exo-treated NAF, promoted endothelial morphogenesis. The Dynamin (DNM) inhibitor Dynasore (Dyn) inhibited LO-uptake, as well as MYC activation and tube formation. Transient silencing of DNM2 significantly reduced LO uptake, suggesting that uptake occurs by phagocytosis. LO treatment increased levels of MYC targets in NAF, suggesting that MYC is involved in LO-induced reprogramming of NAF. Accordingly, MYC expression was higher in activated fibroblasts than NAF, and MYC overexpression in NAF induced hyperplasia in normal prostate epithelium in mice, suggesting MYC activation is an early event in cancer development. Summary/Conclusion: Our results indicate that tumor-derived LO induce a novel, c-MYC mediated, pro-tumorigenic reprogramming of fibroblasts that can be reverted by selectively inhibiting LO uptake.

3.2567 The potential of endurance exercise-derived exosomes to treat metabolic diseases

Safddar, A., Saleemi, A. and Tarnopolsky, M.A.
Nature Endocrinol., ***12(9)***, *504-517 (2016)*

Endurance exercise-mediated multisystemic adaptations are known to mitigate metabolism-related disorders such as obesity and type 2 diabetes mellitus (T2DM). However, the underlying molecular mechanisms that promote crosstalk between organs and orchestrate the pro-metabolic effects of endurance exercise remain unclear. Exercise-induced release of peptides and nucleic acids from skeletal muscle and other organs (collectively termed 'exerkines') has been implicated in mediating these systemic adaptations. Given that the extracellular milieu is probably not a hospitable environment for labile exerkines, a lipid vehicle-based mode of delivery has originated over the course of evolution. Two types of extracellular vesicles, exosomes and microvesicles, have been shown to contain proteins and nucleic acids that participate in a variety of physiological and pathological processes. Exosomes, in particular, have been shown to facilitate the exchange of peptides, microRNA, mRNA and mitochondrial DNA between cells and tissues. Intriguingly, circulatory extracellular vesicle content increases in an intensity-dependant manner in response to endurance exercise. We propose that the systemic benefits of exercise are modulated by exosomes and/or microvesicles functioning in an autocrine, paracrine and/or endocrine manner. Furthermore, we posit that native or modified exosomes, and/or microvesicles enriched with exerkines will have therapeutic utility in the treatment of obesity and T2DM.

3.2568 Activation of lysosomal P2X4 by ATP transported into lysosomes via VNUT/SLC17A9 using V-ATPase generated voltage gradient as the driving force

Zhong, X.Z., Cao, Q., Sun, X. and Dong, X-P.
J. Physiol., ***594(15)***, *4253-4266 (2016)*

The lysosome contains abundant ATP which plays important roles in lysosome functions and in cell signalling. Recently, solute carrier family 17 member 9 (SLC17A9, also known as VNUT for vesicular nucleotide transporter) proteins were suggested to function as a lysosomal ATP transporter responsible for lysosomal ATP accumulation, and P2X4 receptors were suggested to be lysosomal ion channels that are activated by luminal ATP. However, the molecular mechanism of SLC17A9 transporting ATP and the regulatory mechanism of lysosomal P2X4 are largely unknown. In this study, we report that SLC17A9-mediated ATP transport across lysosomal membranes is suppressed by Bafilomycin A1, the V-ATPase inhibitor. By measuring P2X4 activity, which is indicative of ATP transport across lysosomal membranes, we further demonstrated that SLC17A9 mainly uses voltage gradient but not pH gradient as the driving

force to transport ATP into lysosomes. This study provides a molecular mechanism for lysosomal ATP transport mediated by SLC17A9. It also suggests a regulatory mechanism of lysosomal P2X4 by SLC17A9.

3.2569 TMX1 determines cancer cell metabolism as a thiol-based modulator of ER–mitochondria Ca2+ flux
Raturi, A. et al
J. Cell Biol., ***214(4)***, *433-444 (2016)*

The flux of Ca^{2+} from the endoplasmic reticulum (ER) to mitochondria regulates mitochondria metabolism. Within tumor tissue, mitochondria metabolism is frequently repressed, leading to chemotherapy resistance and increased growth of the tumor mass. Therefore, altered ER–mitochondria Ca^{2+} flux could be a cancer hallmark, but only a few regulatory proteins of this mechanism are currently known. One candidate is the redox-sensitive oxidoreductase TMX1 that is enriched on the mitochondria-associated membrane (MAM), the site of ER–mitochondria Ca^{2+} flux. Our findings demonstrate that cancer cells with low TMX1 exhibit increased ER Ca^{2+}, accelerated cytosolic Ca^{2+} clearance, and reduced Ca^{2+} transfer to mitochondria. Thus, low levels of TMX1 reduce ER–mitochondria contacts, shift bioenergetics away from mitochondria, and accelerate tumor growth. For its role in intracellular ER–mitochondria Ca^{2+} flux, TMX1 requires its thioredoxin motif and palmitoylation to target to the MAM. As a thiol-based tumor suppressor, TMX1 increases mitochondrial ATP production and apoptosis progression.

3.2570 Plasma exosome profiles from dairy cows with divergent fertility phenotypes
Mitchell, M.D., Scholz-Romero, K., Ree, S., Peiris, H.N., Koh, Y.Q., Meier, S., Walker, C.G., Burke, C.R., Roche, J.R., Rice, G. and Salomon, C.
J. Dairy Sci., ***99(9)***, *7590-7601 (2016)*

Cell-to-cell communication in physiological and pathological conditions may be influenced by neighboring cells, distant tissues, or local environmental factors. Exosomes are specific subsets of extracellular vesicles that internalize and deliver their content to near and distant sites. Exosomes may play a role in the maternal-embryo crosstalk vital for the recognition and maintenance of a pregnancy; however, their role in dairy cow reproduction has not been established. This study aimed to characterize the exosome profile in the plasma of 2 strains of dairy cow with divergent fertility phenotypes. Plasma was obtained and characterized on the basis of genetic ancestry as fertile (FERT; <23% North American genetics, New Zealand Holstein-Friesian strain, n = 8) or subfertile (SUBFERT; >92% North American genetics, North American Holstein-Friesian strain, n = 8). Exosomes were isolated by differential and buoyant density centrifugation and characterized by size distribution (nanoparticle tracking analysis, NanoSight NS500, NanoSight Ltd., Amesbury, UK), the presence of CD63 (Western blot), and their morphology (electron microscopy). The total number of exosomes was determined by quantifying the immunoreactive CD63 (ExoELISA kit, System Biosciences), and the protein content established by mass spectrometry. Enriched exosome fractions were identified as cup-shape vesicles with diameters around 100 nm and positive for the CD63 marker. The concentration of exosomes was 50% greater in FERT cows. Mass spectrometry identified 104 and 117 proteins in FERT and SUBFERT cows, of which 23 and 36 were unique, respectively. Gene ontology analysis revealed enrichment for proteins involved in immunomodulatory processes and cell-to-cell communication. Although the role of exosomes in dairy cow reproduction remains to be elucidated, their quantification and content in models with divergent fertility phenotypes could provide novel information to support both physiological and genetic approaches to improving dairy cow fertility.

3.2571 Short communication: Proteins from circulating exosomes represent metabolic state in transition dairy cows
Crookenden, M.A., walker, C.G., peiris, H., Koh, Y., Heiser, A., Loor, J.J., Moyes, K.M., Murray, A., Dukkipati, V.S.R., Kay, K., Meier, S., Roche, J.R. and Mitchell, M.D.
J. Dairy Sci., ***99(9)***, *7661-7668 (2016)*

Biomarkers that identify prepathological disease could enhance preventive management, improve animal health and productivity, and reduce costs. Circulating extracellular vesicles, particularly exosomes, are considered to be long-distance, intercellular communication systems in human medicine. Exosomes provide tissue-specific messages of functional state and can alter the cellular activity of recipient tissues through their protein and microRNA content. We hypothesized that exosomes circulating in the blood of cows during early lactation would contain proteins representative of the metabolic state of important tissues, such as liver, which play integral roles in regulating the physiology of cows postpartum. From a total of 150 cows of known metabolic phenotype, 10 cows were selected with high (n = 5; high risk) and

low (n = 5; low risk) concentrations of nonesterified fatty acids, β-hydroxybutyrate, and liver triacylglycerol during wk 1 and 2 after calving. Exosomes were extracted from blood on the day of calving (d 0) and postcalving at wk 1 and wk 4, and their protein composition was determined by mass spectroscopy. Extracellular vesicle protein concentration and the number of exosome vesicles were not affected by risk category; however, the exosome protein cargo differed between the groups, with proteins at each time point identified as being unique to the high- and low-risk groups. The proteins α-2 macroglobulin, fibrinogen, and oncoprotein-induced transcript 3 were unique to the high-risk cows on d 0 and have been associated with metabolic syndrome and liver function in humans. Their presence may indicate a more severe inflammatory state and a greater degree of liver dysfunction in the high-risk cows than in the low-risk cows, consistent with the high-risk cows' greater plasma β-hydroxybutyrate and liver triacylglycerol concentrations. The commonly shared proteins and those unique to the low-risk category indicate a role for exosomes in immune function. The data provide preliminary evidence of a potential role for exosomes in the immune function in transition dairy cows and exosomal protein cargo as biomarkers of metabolic state.

3.2572 The coming of age of the mitochondria–ER contact: a matter of thickness
Giacomello, M. and Pellegrini, L.
*Cell Death and Differentiation, **23(9)**, 1417-1427 (2016)*

The sites of near-contact between the mitochondrion and the endoplasmic reticulum (ER) have earned a lot of attention due to their key role in the maintenance of lipid and calcium (Ca^{2+}) homeostasis, in the initiation of autophagy and mitochondrial division, and in sensing metabolic shifts. At these sites, typically called MAMs (mitochondria-associated ER membranes) or MERCs (mitochondria–ER contacts), the organelles juxtapose at a distance that can range from ~10 to ~50 nm. The multifunctional role of this subcellular compartment is puzzling; further, recent studies have shown that mitochondria–ER contacts are highly plastic structures that remodel upon metabolic transitions and that their activity in controlling lipid homeostasis could be involved in Alzheimer's disease pathogenesis. This review aims at integrating the functions of this subcellular compartment to its most characterizing and unexplored structural parameter, their 'thickness': that is, the width of the cleft that separates the cytosolic face of the outer mitochondrial membrane from that of the ER. We describe and discuss the reasons why the thickness of a MERC should be considered a regulated structural parameter of the cell that defines and controls its function. Further, we propose a MERC classification that will help organize the expanding field of MERCs biology and of their role in cell physiology and human disease.

3.2573 N-Heterocyclic Carbene–Polyethylenimine Platinum Complexes with Potent in Vitro and in Vivo Antitumor Efficacy
Chekkat, N., Dahm, G., Chardon, E., Wantz, M., Sitz, J., Decossas, M., Lambert, O., Frisch, B., Rubbiani, R., Gasser, G., Guichard, G., Fournel, S. and Bellemin-Laponnaz, S.
*Bioconjugate Chem., **27(8)**, 1942-1948 (2016)*

The current interest for platinum N-heterocyclic carbene complexes in cancer research stems from their impressive toxicity reported against a range of different human cancer cells. To date, the demonstration of their in vivo efficacy relative to that of established platinum-based drugs has not been specifically addressed. Here, we introduce an innovative approach to increase the NHC-Pt complex potency whereby multiple NHC-Pt(II) complexes are coordinated along a polyethylenimine polymer (PEI) chain. We show that such NHC-Pt(II)-PEI conjugates induce human cancer cell death in vitro and in vivo in a xenograft mouse model with no observable side effects in contrast to oxaliplatin. Additional studies indicate nucleus and mitochondria targeting and suggest various mechanisms of action compared to classical platinum-based anticancer drugs.

3.2574 Lysosomal Acid Lipase Hydrolyzes Retinyl Ester and Affects Retinoid Turnover
Grumet, L. et al
*J. Biol. Chem., **291(34)**, 17977-17987 (2016)*

Lysosomal acid lipase (LAL) is essential for the clearance of endocytosed cholesteryl ester and triglyceride-rich chylomicron remnants. Humans and mice with defective or absent LAL activity accumulate large amounts of cholesteryl esters and triglycerides in multiple tissues. Although chylomicrons also contain retinyl esters (REs), a role of LAL in the clearance of endocytosed REs has not been reported. In this study, we found that murine LAL exhibits RE hydrolase activity. Pharmacological inhibition of LAL in the human hepatocyte cell line HepG2, incubated with chylomicrons, led to increased

accumulation of REs in endosomal/lysosomal fractions. Furthermore, pharmacological inhibition or genetic ablation of LAL in murine liver largely reduced *in vitro* acid RE hydrolase activity. Interestingly, LAL-deficient mice exhibited increased RE content in the duodenum and jejunum but decreased RE content in the liver. Furthermore, LAL-deficient mice challenged with RE gavage exhibited largely reduced post-prandial circulating RE content, indicating that LAL is required for efficient nutritional vitamin A availability. In summary, our results indicate that LAL is the major acid RE hydrolase and required for functional retinoid homeostasis.

3.2575 Dehydroepiandrosterone-induced changes in mitochondrial proteins contribute to phenotypic alterations in hepatoma cells

Cheng, M-L., Chi, L-M., Wu, P-R. and Ho, H-Y.
Biochem. Pharmacol., 117, 20-34 (2016)

Dehydroepiandrosterone (DHEA)-induced growth arrest of hepatoma cells is associated with metabolic disturbance. Our previous study has suggested that DHEA may cause cellular energy drain. It is possible that mitochondrial dysfunction may be mechanistically implicated in DHEA-induced changes in cellular phenotype. Treatment of SK-Hep-1 cells with DHEA caused significant reduction in proliferation, colony formation, and growth in semi-solid medium. Such changes in cellular phenotype were associated with mitochondrial depolarization, increase in mitochondrial mass, and decrease in respiratory activity. Level of reactive oxygen species (ROS) increased in DHEA-treated cells. To explore the mechanistic aspect of DHEA-induced mitochondrial dysfunction, we employed SILAC approach to study the changes in the mitoproteome of SK-Hep-1 cells after DHEA treatment. Respiratory chain complex proteins such as NDUFB8 and SDHB were differentially expressed. Of mitochondrial proteins with altered expression, FAST kinase domain-containing protein 2 (FASTKD2) showed significantly reduced expression. Exogenous expression of FASTKD2 in SK-Hep-1 cells increased their resistance to growth-inhibitory effect of DHEA, though it alone did not affect cell growth. FASTKD2 expression partially reversed the effect of DHEA on mitochondria, and reduced DHEA-induced ROS generation. Our results suggest that DHEA induces changes in mitochondrial proteins and respiratory activity, and contributes to growth arrest. FASTKD2 may be an important regulator of mitochondrial physiology, and represent a downstream target for DHEA.

3.2576 The Golgi apparatus acts as a platform for TBK1 activation after viral RNA sensing

Pourcelot, M., Zemirli, N., Da Costa, L.S., Loyant, R., Garcin, D., Vitour, D., Munitic, I., Vazquez, A. and Arnoult, D.
*BMC Biology, **14**:69 (2016)*

Background
After viral infection and the stimulation of some pattern-recognition receptors, TANK-binding kinase I (TBK1) is activated by K63-linked polyubiquitination followed by *trans*-autophosphorylation. While the activated TBK1 induces type I interferon production by phosphorylating the transcription factor IRF3, the precise molecular mechanisms underlying TBK1 activation remain unclear.
Results
We report here the localization of the ubiquitinated and phosphorylated active form of TBK1 to the Golgi apparatus after the stimulation of RIG-I-like receptors (RLRs) or Toll-like receptor-3 (TLR3), due to TBK1 K63-linked ubiquitination on lysine residues 30 and 401. The ubiquitin-binding protein optineurin (OPTN) recruits ubiquitinated TBK1 to the Golgi apparatus, leading to the formation of complexes in which TBK1 is activated by *trans*-autophosphorylation. Indeed, OPTN deficiency in various cell lines and primary cells impairs TBK1 targeting to the Golgi apparatus and its activation following RLR or TLR3 stimulation. Interestingly, the Bluetongue virus NS3 protein binds OPTN at the Golgi apparatus, neutralizing its activity and thereby decreasing TBK1 activation and downstream signaling.
Conclusions
Our results highlight an unexpected role of the Golgi apparatus in innate immunity as a key subcellular gateway for TBK1 activation after RNA virus infection.

3.2577 Characterisation of mouse epididymosomes reveals a complex profile of microRNAs and a potential mechanism for modification of the sperm epigenome

Reilly, J.N., McLaughlin, E.A., Stanger, S.J., Anderson, A.L., Hutcheon, K., Church, K., Mihalas, B.P., Tyagi, S., Holt, J.E., Eamens, A.L. and Nixon, B,
*Scientific Reports, **6**:31794 (2016)*

Recent evidence has shown that the sperm epigenome is vulnerable to dynamic modifications arising from a variety of paternal environment exposures and that this legacy can serve as an important determinant of intergenerational inheritance. It has been postulated that such exchange is communicated to maturing spermatozoa via the transfer of small non-protein-coding RNAs (sRNAs) in a mechanism mediated by epididymosomes; small membrane bound vesicles released by the soma of the male reproductive tract (epididymis). Here we confirm that mouse epididymosomes encapsulate an impressive cargo of >350 microRNAs (miRNAs), a developmentally important sRNA class, the majority (~60%) of which are also represented by the miRNA signature of spermatozoa. This includes >50 miRNAs that were found exclusively in epididymal sperm and epididymosomes, but not in the surrounding soma. We also documented substantial changes in the epididymosome miRNA cargo, including significant fold changes in almost half of the miRNAs along the length of the epididymis. Finally, we provide the first direct evidence for the transfer of several prominent miRNA species between mouse epididymosomes and spermatozoa to afford novel insight into a mechanism of intercellular communication by which the sRNA payload of sperm can be selectively modified during their post-testicular maturation.

3.2578 Isolation of Intact and Functional Melanosomes from the Retinal Pigment Epithelium
Pelkonen, L., Reinsalo, M., Morin-Picardat, E., Kidron, H. and Urtti, A.
*PloS One, **11(8)**, e0160352 (2016)*

Melanosomes of retinal pigment epithelium (RPE) have many vision supporting functions. Melanosome research would benefit from a method to isolate pure and characterized melanosomes. Sucrose gradient centrifugation is the most commonly used method for isolation of RPE melanosomes, but the isolated products are insufficiently characterized and their quality is unclear. Here we introduce a new gentle method for fractionation of porcine RPE that produces intact functional melanosomes with minimal cross-contamination from other cell organelles. The characterization of isolated organelles was conducted with several methods confirming the purity of the isolated melanosomal fraction (transmission electron microscopy, immunoblotting) and presence of the melanosomal membrane (fluorescence staining of melanosomal membrane, zeta potential measurement). We demonstrate that our isolation method produces RPE melanosomes with the ability to generate free phosphate (Pi) from ATP thereby proving that many membrane proteins remain functional after isolation. The isolated porcine RPE melanosomes represented V-type H+ATPase activity that was demonstrated with bafilomycin A1, a specific V-ATPase inhibitor. We anticipate that the isolation method described here can easily be optimized for the isolation of stage IV melanosomes from other pigmented cell types and tissues.

3.2579 Tylophorine Analog DCB-3503 Inhibited Cyclin D1 Translation through Allosteric Regulation of Heat Shock Cognate Protein 70
Wang, Y., Lam, W., Chen, S-R., Guan, F-L., Dutchman, G.E., Francis, s., Baker, D.C. and Cheng, Y-C.
*Scientific Reports, **6**:32832 (2016)*

Tylophorine analog DCB-3503 is a potential anticancer and immunosuppressive agent that suppresses the translation of cellular regulatory proteins, including cyclin D1, at the elongation step. However, the molecular mechanism underlying this phenomenon remains unknown. This study demonstrates that DCB-3503 preferentially binds to heat shock cognate protein 70 (HSC70), which is a determinant for cyclin D1 translation by binding to the 3′-untranslated region (3′ UTR) of its mRNA. DCB-3503 allosterically regulates the ATPase and chaperone activities of HSC70 by promoting ATP hydrolysis in the presence of specific RNA binding motifs (AUUUA) of cyclin D1 mRNA. The suppression of cyclin D1 translation by DCB-3503 is not solely caused by perturbation of the homeostasis of microRNAs, although the microRNA processing complex is dissociated with DCB-3503 treatment. This study highlights a novel regulatory mechanism of protein translation with AUUUA motifs in the 3′ UTR of mRNA by HSC70, and its activity can be allosterically modulated by DCB-3503. DCB-3503 may be used to treat malignancies, such as hepatocellular carcinoma or breast cancer with elevated expression of cyclin D1.

3.2580 Restriction of Aerobic Metabolism by Acquired or Innate Arylsulfatase B Deficiency: A New Approach to the Warburg Effect
Bhattacharyya, S., Feferman, L. and Tobocman, J.K.
*Scientific Reports, **6**:32885 (2016)*

Aerobic respiration is required for optimal efficiency of metabolism in mammalian cells. Under circumstances when oxygen utilization is impaired, cells survive by anerobic metabolism. The malignant cell has cultivated the use of anerobic metabolism in an aerobic environment, the Warburg effect, but the

explanation for this preference is not clear. This paper presents evidence that deficiency of the enzyme arylsulfatase B (ARSB; N-acetylgalactosamine 4-sulfatase), either innate or acquired, helps to explain the Warburg phenomenon. ARSB is the enzyme that removes 4-sulfate groups from the non-reducing end of chondroitin 4-sulfate and dermatan sulfate. Previous reports indicated reduced ARSB activity in malignancy and replication of the effects of hypoxia by decline in ARSB. Hypoxia reduced ARSB activity, since molecular oxygen is needed for post-translational modification of ARSB. In this report, studies were performed in human HepG2 cells and in hepatocytes from ARSB-deficient and normal C57BL/6J control mice. Decline of ARSB, in the presence of oxygen, profoundly reduced the oxygen consumption rate and increased the extracellular acidification rate, indicating preference for aerobic glycolysis. Specific study findings indicate that decline in ARSB activity enhanced aerobic glycolysis and impaired normal redox processes, consistent with a critical role of ARSB and sulfate reduction in mammalian metabolism.

3.2581 Functional Study and Mapping Sites for Interaction with the Target Enzyme in Retinal Degeneration 3 (RD3) Protein
Peshenko, I.V., Olshevskaya, E.V. and Dizhoor, A.M.
J. Biol. Chem., ***291(37)***, *19713-19723 (2016)*

Retinal degeneration 3 (RD3) protein, essential for normal expression of retinal membrane guanylyl cyclase (RetGC) in photoreceptor cells, blocks RetGC catalytic activity and stimulation by guanylyl cyclase-activating proteins (GCAPs). In a mouse retina, RD3 inhibited both RetGC1 and RetGC2 isozymes. Photoreceptors in the *rd3/rd3* mouse retinas lacking functional RD3 degenerated more severely than in the retinas lacking both RetGC isozymes, consistent with a hypothesis that the inhibitory activity of RD3 has a functional role in photoreceptors. To map the potential target-binding site(s) on RD3, short evolutionary conserved regions of its primary structure were scrambled and the mutations were tested for the RD3 ability to inhibit RetGC1 and co-localize with the cyclase in co-transfected cells. Substitutions in 4 out of 22 tested regions, ^{87}KIHP^{90}, ^{93}CGPAI^{97}, ^{99}RFRQ^{102}, and ^{119}RSVL^{122}, reduced the RD3 apparent affinity for the cyclase 180–700-fold. Changes of amino acid sequences outside the Lys^{87}–Leu^{122} central portion of the molecule either failed to prevent RD3 binding to the cyclase or had a much smaller effect. Mutations in the ^{93}CGPAI^{97} portion of a predicted central α-helix most drastically suppressed the inhibitory activity of RD3 and disrupted RD3 co-localization with RetGC1 in HEK293 cells. Different side chains replacing Cys^{93} profoundly reduced RD3 affinity for the cyclase, irrespective of their relative helix propensities. We conclude that the main RetGC-binding interface on RD3 required for the negative regulation of the cyclase localizes to the Lys^{87}–Leu^{122} region.

3.2582 Microvesicles from brain-extract—treated mesenchymal stem cells improve neurological functions in a rat model of ischemic stroke
Lee, J.Y., Kim, E., Choi, S-M., Kim, D-W., Kim, K.P., Lee, I. and Kimm, H-S.
*Scientific Reports, **6**:33038 (2016)*

Transplantation of mesenchymal stem cells (MSCs) was reported to improve functional outcomes in a rat model of ischemic stroke, and subsequent studies suggest that MSC-derived microvesicles (MVs) can replace the beneficial effects of MSCs. Here, we evaluated three different MSC-derived MVs, including MVs from untreated MSCs (MSC-MVs), MVs from MSCs treated with normal rat brain extract (NBE-MSC-MVs), and MVs from MSCs treated with stroke-injured rat brain extract (SBE-MSC-MVs), and tested their effects on ischemic brain injury induced by permanent middle cerebral artery occlusion (pMCAO) in rats. NBE-MSC-MVs and SBE-MSC-MVs had significantly greater efficacy than MSC-MVs for ameliorating ischemic brain injury with improved functional recovery. We found similar profiles of key signalling proteins in NBE-MSC-MVs and SBE-MSC-MVs, which account for their similar therapeutic efficacies. Immunohistochemical analyses suggest that brain-extract—treated MSC-MVs reduce inflammation, enhance angiogenesis, and increase endogenous neurogenesis in the rat brain. We performed mass spectrometry proteomic analyses and found that the total proteomes of brain-extract—treated MSC-MVs are highly enriched for known vesicular proteins. Notably, MSC-MV proteins upregulated by brain extracts tend to be modular for tissue repair pathways. We suggest that MSC-MV proteins stimulated by the brain microenvironment are paracrine effectors that enhance MSC therapy for stroke injury.

3.2583 Chromatin remodeling during the in vivo glial differentiation in early Drosophila embryos
Ye, Y., Gu, L., Chen, X., Shi, J., Zhang, X. and Jiang, C.
*Scientific Reports, **6**:33422 (2016)*

Chromatin remodeling plays a critical role in gene regulation and impacts many biological processes. However, little is known about the relationship between chromatin remodeling dynamics and in vivo cell lineage commitment. Here, we reveal the patterns of histone modification change and nucleosome positioning dynamics and their epigenetic regulatory roles during the in vivo glial differentiation in early Drosophila embryos. The genome-wide average H3K9ac signals in promoter regions are decreased in the glial cells compared to the neural progenitor cells. However, H3K9ac signals are increased in a group of genes that are up-regulated in glial cells and involved in gliogenesis. There occurs extensive nucleosome remodeling including shift, loss, and gain. Nucleosome depletion regions (NDRs) form in both promoters and enhancers. As a result, the associated genes are up-regulated. Intriguingly, NDRs form in two fashions: nucleosome shift and eviction. Moreover, the mode of NDR formation is independent of the original chromatin state of enhancers in the neural progenitor cells.

3.2584 Quantitative Lipid Droplet Proteome Analysis Identifies Annexin A3 as a Cofactor for HCV Particle Production
Rösch, K., Kwiatkowski, M., Hofman, S. et al
*Cell Reports, **16**, 3219-3231 (2016)*

Lipid droplets are vital to hepatitis C virus (HCV) infection as the putative sites of virion assembly, but morphogenesis and egress of virions remain ill defined. We performed quantitative lipid droplet proteome analysis of HCV-infected cells to identify co-factors of that process. Our results demonstrate that HCV disconnects lipid droplets from their metabolic function. Annexin A3 (ANXA3), a protein enriched in lipid droplet fractions, strongly impacted HCV replication and was characterized further: ANXA3 is recruited to lipid-rich fractions in HCV-infected cells by the viral core and NS5A proteins. ANXA3 knockdown does not affect HCV RNA replication but severely impairs virion production with lower specific infectivity and higher density of secreted virions. ANXA3 is essential for the interaction of viral envelope E2 with apolipoprotein E (ApoE) and for trafficking, but not lipidation, of ApoE in HCV-infected cells. Thus, we identified ANXA3 as a regulator of HCV maturation and egress.

3.2585 Interaction of the Human Papillomavirus E6 Oncoprotein with Sorting Nexin 27 Modulates Endocytic Cargo Transport Pathways
Ganti, K., Massimi, P., Monzo-merino, J., Tomaic, V., Pim, D., Playford, M.P., Lizano, M., Roberts, S., Kranjec, C., Doorbar, J. and Banks, L.
*PloS Pathogens, **12(9)**, e1005854 (2016)*

A subset of high-risk Human Papillomaviruses (HPVs) are the causative agents of a large number of human cancers, of which cervical is the most common. Two viral oncoproteins, E6 and E7, contribute directly towards the development and maintenance of malignancy. A characteristic feature of the E6 oncoproteins from cancer-causing HPV types is the presence of a PDZ binding motif (PBM) at its C-terminus, which confers interaction with cellular proteins harbouring PDZ domains. Here we show that this motif allows E6 interaction with Sorting Nexin 27 (SNX27), an essential component of endosomal recycling pathways. This interaction is highly conserved across E6 proteins from multiple high-risk HPV types and is mediated by a classical PBM-PDZ interaction but unlike many E6 targets, SNX27 is not targeted for degradation by E6. Rather, in HPV-18 positive cell lines the association of SNX27 with components of the retromer complex and the endocytic transport machinery is altered in an E6 PBM-dependent manner. Analysis of a SNX27 cargo, the glucose transporter GLUT1, reveals an E6-dependent maintenance of GLUT1 expression and alteration in its association with components of the endocytic transport machinery. Furthermore, knockdown of E6 in HPV-18 positive cervical cancer cells phenocopies the loss of SNX27, both in terms of GLUT1 expression levels and its vesicular localization, with a concomitant marked reduction in glucose uptake, whilst loss of SNX27 results in slower cell proliferation in low nutrient conditions. These results demonstrate that E6 interaction with SNX27 can alter the recycling of cargo molecules, one consequence of which is modulation of nutrient availability in HPV transformed tumour cells.

3.2586 Immunomodulatory effects of exosomes produced by virus-infected cells
Petrik, J.
Transfusion and Apheresis Science, 55, 84-91 (2016)

Viruses have developed a spectrum of ways to modify cellular pathways to hijack the cell machinery for the synthesis of their nucleic acid and proteins. Similarly, they use intracellular vesicular mechanisms of trafficking for their assembly and eventual release, with a number of viruses acquiring their envelope from

internal or plasma cell membranes. There is an increasing number of reports on viral exploitation of cell secretome pathways to avoid recognition and stimulation of the immune response. Extracellular vesicles (EV) containing viral particles have been shown to shield viruses after exiting the host cell, in some cases challenging the boundaries between viral groups traditionally characterised as enveloped and non-enveloped. Apart from viral particles, EV can spread the virus also carrying viral genome and can modify the target cells through their cargo of virus-coded miRNAs and proteins as well as selectively packaged cellular mRNAs, miRNAs, proteins and lipids, differing in composition and quantities from the cell of origin.

3.2587 Localization, Shedding, Regulation and Function of Aminopeptidase N/CD13 on Fibroblast like Synoviocytes

Morgan, R.L., Behbahani-Nejad, N., Endres, J., Amin, M.A., Lepore, N.J:, Du, Y., Urqehart, A., Chung, K.C. and Fox, D.A.
*PloS One, **11(9)**, e0162008 (2016)*

Aminopeptidase N/CD13 is highly expressed by fibroblast like synoviocytes (FLS) and may play a role in rheumatoid arthritis (RA). CD13 was previously detected in human synovial fluid where it was significantly increased in RA compared to osteoarthritis. In this study we found that CD13 in biological fluids (plasma, synovial fluid, FLS culture supernatant) is present as both a soluble molecule and on extracellular vesicles, including exosomes, as assessed by differential ultracentrifugation and density gradient separation. Having determined CD13 could be released as a soluble molecule from FLS, we examined potential mechanisms by which CD13 might be shed from the FLS membrane. The use of protease inhibitors revealed that CD13 is cleaved from the FLS surface by metalloproteinases. siRNA treatment of FLS revealed one of those proteases to be MMP14. We determined that pro-inflammatory cytokines (TNFα, IFNγ, IL-17) upregulated CD13 mRNA in FLS, which may contribute to the increased CD13 in RA synovium and synovial fluid. Inhibition of CD13 function by either inhibitors of enzymatic activity or anti-CD13 antibodies resulted in decreased growth and diminished migration of FLS. This suggests that CD13 may be involved in the pathogenic hyperplasia of RA FLS. This data expands potential roles for CD13 in the pathogenesis of RA.

3.2588 Vimentin, a Novel NF-κB Regulator, Is Required for Meningitic Escherichia coli K1-Induced Pathogen Invasion and PMN Transmigration across the Blood-Brain Barrier

Huang, S-H., Chi, F., Peng, L., Bo, T., Zhang, B., Liu, L-Q., Wu, X., Mor-Vaknin, N., Markovitz, D.M., Cao, H.and Zhou, Y-H.
*PloS One, **11(9)**, e0162641 (2016)*

Background
NF-κB activation, pathogen invasion, polymorphonuclear leukocytes (PMN) transmigration (PMNT) across the blood-brain barrier (BBB) are the pathogenic triad hallmark features of bacterial meningitis, but the mechanisms underlying these events remain largely unknown. Vimentin, which is a novel NF-κB regulator, is the primary receptor for the major Escherichia coli K1 virulence factor IbeA that contributes to the pathogenesis of neonatal bacterial sepsis and meningitis (NSM). We have previously shown that IbeA-induced NF-κB signaling through its primary receptor vimentin as well as its co-receptor PTB-associated splicing factor (PSF) is required for pathogen penetration and leukocyte transmigration across the BBB. This is the first in vivo study to demonstrate how vimentin and related factors contributed to the pathogenic triad of bacterial meningitis.
Methodology/Principal Findings
The role of vimentin in IbeA+ E. coli K1-induced NF-κB activation, pathogen invasion, leukocyte transmigration across the BBB has now been demonstrated by using vimentin knockout (KO) mice. In the in vivo studies presented here, IbeA-induced NF-κB activation, E. coli K1 invasion and polymorphonuclear neutrophil (PMN) transmigration across the BBB were significantly reduced in Vim-/- mice. Decreased neuronal injury in the hippocampal dentate gyrus was observed in Vim-/- mice with meningitis. The major inflammatory regulator α7 nAChR and several signaling molecules contributing to NF-κB activation (p65 and p-CamKII) were significantly reduced in the brain tissues of the Vim-/- mice with E. coli meningitis. Furthermore, Vim KO resulted in significant reduction in neuronal injury and in α7 nAChR-mediated calcium signaling.
Conclusion/Significance
Vimentin, a novel NF-κB regulator, plays a detrimental role in the host defense against meningitic infection by modulating the NF-κB signaling pathway to increase pathogen invasion, PMN recruitment,

BBB permeability and neuronal inflammation. Our findings provide the first evidence for Vim-dependent mechanisms underlying the pathogenic triad of bacterial meningitis.

3.2589 Isolation of human trophoblastic extracellular vesicles and characterization of their cargo and antiviral activity

Ouyang, Y., Bayer, A., Chu, T., Tyurin, V., Kagan, V., Morelli, A.E., Coyne, C.B. and Sadovsky, Y.
*Placenta, **47**, 86-95 (2016)*

Introduction
Primary human trophoblasts release a repertoire of extracellular vesicles (EVs). Among them are nano-sized exosomes, which we found to suppress the replication of a wide range of diverse viruses. These exosomes contain trophoblastic microRNAs (miRNAs) that are expressed from the chromosome 19 miRNA cluster and exhibit antiviral properties. Here, we report our investigation of the cargo of placental EVs, focusing on the composition and the antiviral properties of exosomes, microvesicles, and apoptotic blebs.
Methods
We isolated EVs using ultracentrifugation and defined their purity using immunoblotting, electron microscopy, and nanoparticle tracking. We used liquid chromatography-electrospray ionization-mass spectrometry, protein mass spectrometry, and miRNA TaqMan card PCR to examine the phospholipids, proteins, and miRNA cargo of trophoblastic EVs and an *in vitro* viral infection assay to assess the antiviral properties of EVs.
Results
We found that all three EV types contain a comparable repertoire of miRNA. Interestingly, trophoblastic exosomes harbor a protein and phospholipid profile that is distinct from that of microvesicles or apoptotic blebs. Functionally, trophoblastic exosomes exhibit the highest antiviral activity among the EVs. Consistently, plasma exosomes derived from pregnant women recapitulate the antiviral effect of trophoblastic exosomes derived from *in vitro* cultures of primary human trophoblasts.
Discussion
When compared to other trophoblastic EVs, exosomes exhibit a unique repertoire of proteins and phospholipids, but not miRNAs, and a potent viral activity. Our work suggests that human trophoblastic EVs may play a key role in maternal-placental-fetal communication.

3.2590 A novel affinity-based method for the isolation of highly purified extracellular vesicles

Nakai, W., Yoshida, T., Diez, D., Miyatake, Y., Nishibu, T., Imawaka, N., Narusem K., Sadamura, Y. and Hanayama, R.
*Scientifica Reports, **6**:33935 (2016)*

Extracellular vesicles (EVs) such as exosomes and microvesicles serve as messengers of intercellular network, allowing exchange of cellular components between cells. EVs carry lipids, proteins, and RNAs derived from their producing cells, and have potential as biomarkers specific to cell types and even cellular states. However, conventional methods (such as ultracentrifugation or polymeric precipitation) for isolating EVs have disadvantages regarding purity and feasibility. Here, we have developed a novel method for EV purification by using Tim4 protein, which specifically binds the phosphatidylserine displayed on the surface of EVs. Because the binding is Ca2+-dependent, intact EVs can be easily released from Tim4 by adding Ca2+ chelators. Tim4 purification, which we have applied to cell conditioned media and biofluids, is capable of yielding EVs of a higher purity than those obtained using conventional methods. The lower contamination found in Tim4-purified EV preparations allows more EV-specific proteins to be detected by mass spectrometry, enabling better characterization and quantification of different EV populations' proteomes. Tim4 protein can also be used as a powerful tool for quantification of EVs in both ELISA and flow cytometry formats. Thus, the affinity of Tim4 for EVs will find abundant applications in EV studies.

3.2591 Phosphatidic Acid Produced by RalA-activated PLD2 Stimulates Caveolae-mediated Endocytosis and Trafficking in Endothelial Cells

Jiang, Y., Sverdlov, M.S., Toth, P.T., Huang, L.S., Du, G., Liu, Y., Natarajan, V. and Minshall, R.D:
*J. Biol. Chem., **291(39)**, 20729-20738 (2016)*

Caveolae are the primary route for internalization and transendothelial transport of macromolecules, such as insulin and albumin. Caveolae-mediated endocytosis is activated by Src-dependent caveolin-1 (Cav-1) phosphorylation and subsequent recruitment of dynamin-2 and filamin A (FilA), which facilitate vesicle

fission and trafficking, respectively. Here, we tested the role of RalA and phospholipase D (PLD) signaling in the regulation of caveolae-mediated endocytosis and trafficking. The addition of albumin to human lung microvascular endothelial cells induced the activation of RalA within minutes, and siRNA-mediated down-regulation of RalA abolished fluorescent BSA uptake. Co-immunoprecipitation studies revealed that albumin induced the association between RalA, Cav-1, and FilA; however, RalA knockdown with siRNA did not affect FilA recruitment to Cav-1, suggesting that RalA was not required for FilA and Cav-1 complex formation. Rather, RalA probably facilitates caveolae-mediated endocytosis by activating downstream effectors. PLD2 was shown to be activated by RalA, and inhibition of PLD2 abolished Alexa-488-BSA uptake, indicating that phosphatidic acid (PA) generated by PLD2 may facilitate caveolae-mediated endocytosis. Furthermore, using a PA biosensor, GFP-PASS, we observed that BSA induced an increase in PA co-localization with Cav-1-RFP, which could be blocked by a dominant negative PLD2 mutant. Total internal reflection fluorescence microscopy studies of Cav-1-RFP also showed that fusion of caveolae with the basal plasma membrane was dependent on PLD2 activity. Thus, our results suggest that the small GTPase RalA plays an important role in promoting invagination and trafficking of caveolae, not by potentiating the association between Cav-1 and FilA but by stimulating PLD2-mediated generation of phosphatidic acid.

3.2592 Human Choline Kinase-α Promotes Hepatitis C Virus RNA Replication through Modulation of Membranous Viral Replication Complex Formation

Wong, M-T. and Chen, S.S.

J. Virol., ***90(20)***, *9075-9095 (2016)*

Hepatitis C virus (HCV) infection reorganizes cellular membranes to create an active viral replication site named the membranous web (MW). The role that human choline kinase-α (hCKα) plays in HCV replication remains elusive. Here, we first showed that hCKα activity, not the CDP-choline pathway, promoted viral RNA replication. Confocal microscopy and subcellular fractionation of HCV-infected cells revealed that a small fraction of hCKα colocalized with the viral replication complex (RC) on the endoplasmic reticulum (ER) and that HCV infection increased hCKα localization to the ER. In the pTM-NS3-NS5B model, NS3-NS5B expression increased the localization of the wild-type, not the inactive D288A mutant, hCKα on the ER, and hCKα activity was required for effective trafficking of hCKα and NS5A to the ER. Coimmunoprecipitation showed that hCKα was recruited onto the viral RC presumably through its binding to NS5A domain 1 (D1). hCKα silencing or treatment with CK37, an hCKα activity inhibitor, abolished HCV-induced MW formation. In addition, hCKα depletion hindered NS5A localization on the ER, interfered with NS5A and NS5B colocalization, and mitigated NS5A-NS5B interactions but had no apparent effect on NS5A-NS4B and NS4B-NS5B interactions. Nevertheless, hCKα activity was not essential for the binding of NS5A to hCKα or NS5B. These findings demonstrate that hCKα forms a complex with NS5A and that hCKα activity enhances the targeting of the complex to the ER, where hCKα protein, not activity, mediates NS5A binding to NS5B, thereby promoting functional membranous viral RC assembly and viral RNA replication.

3.2593 The impact of various preanalytical treatments on the phenotype of small extracellular vesicles in blood analyzed by protein microarray

Bæk, R., Søndergaard, E.K.L., varming, K. and Jørgensen, M.M.

J. Immunol. Methods, ***438***, *11-20 (2016)*

The research field of extracellular vesicles (EVs) is increasing immensely and the potential uses of EVs seem endless. They are found in large numbers in various body fluids, and blood samples may well serve as liquid biopsies. However, these small membrane-derived entities of cellular origin are not straightforward to work with in regard to isolation and characterization.
A broad range of relevant preanalytical issues was tested, with a focus on the phenotypic impact of smaller EVs. The influences of the i) blood collection tube used, ii) incubation time before the initial centrifugation, iii) transportation/physical stress, iv) storage temperature and time (short term and long term), v) choice of centrifugation protocol, vi) freeze-thaw cycles, and vii) exosome isolation procedure (ExoQuick™) were examined. To identify the impact of the preanalytical treatments, the relative amounts (detected signal intensities of CD9-, CD63- and/or CD81-positive) and phenotypes of small EVs were analyzed using the multiplexed antibody-based microarray technology, termed the EV Array. The analysis encompassed 15 surface- or surface-related markers, including CD9, CD63, CD81, CD142, and Annexin V.
This study revealed that samples collected in different blood collection tubes suffered to varying degrees from the preanalytical treatments tested here. There is no unequivocal answer to the questions asked. However, in general, the period of time and prospective transportation before the initial centrifugation,

choice of centrifugation protocol, and storage temperature were observed to have major impacts on the samples. On the contrary, long-term storage and freeze-thawing seemed to not have a critical influence. Hence, there are pros and cons of any choice regarding sample collection and preparation and may very well be analysis dependent. However, to compare samples and results, it is important to ensure that all samples are of the same type and have been handled similarly.

3.2594 Membrane Binding by CHMP7 Coordinates ESCRT-III-Dependent Nuclear Envelope Reformation
Olmos, Y., Perdrix-Rosell, A. and Carlton, J.G.
*Current Biology, **26**, 2635-2641 (2016)*

In addition to its role in membrane abscission during cytokinesis, viral budding, endosomal sorting, and plasma membrane repair [1], the endosomal sorting complex required for transport-III (ESCRT-III) machinery has recently been shown to seal holes in the reforming nuclear envelope (NE) during mitotic exit [2 and 3]. ESCRT-III also acts during interphase to repair the NE upon migration-induced rupture [4 and 5], highlighting its key role as an orchestrator of membrane integrity at this organelle. While NE localization of ESCRT-III is dependent upon the ESCRT-III component CHMP7 [3], it is unclear how this complex is able to engage nuclear membranes. Here we show that the N terminus of CHMP7 acts as a novel membrane-binding module. This membrane-binding ability allows CHMP7 to bind to the ER, an organelle continuous with the NE, and it provides a platform to direct NE recruitment of ESCRT-III during mitotic exit. CHMP7's N terminus comprises tandem Winged-Helix domains [6], and, by using homology modeling and structure-function analysis, we identify point mutations that disrupt membrane binding and prevent both ER localization of CHMP7 and its subsequent enrichment at the reforming NE. These mutations also prevent assembly of downstream ESCRT-III components at the reforming NE and proper establishment of post-mitotic nucleo-cytoplasmic compartmentalization. These data identify a novel membrane-binding activity within an ESCRT-III subunit that is essential for post-mitotic nuclear regeneration.

3.2595 Analysis of Yeast Extracellular Vesicles
Rodrigues, M.L., Oliveira, D.L., vargas, G., Girard-Dias, W., Franzen, A.J., Frases, S., Miranda, K. and Nimrichter, L.
*Methods in Mol. Biol., **1459**, 175-190 (2016)*

Extracellular vesicles (EV) are important carriers of biologically active components in a number of organisms, including fungal cells. Experimental characterization of fungal EVs suggested that these membranous compartments are likely involved in the regulation of several biological events. In fungal pathogens, these events include mechanisms of disease progression and/or control, suggesting potential targets for therapeutic intervention or disease prophylaxis. In this manuscript we describe methods that have been used in the last 10 years for the characterization of EVs produced by yeast forms of several fungal species. Experimental approaches detailed in this chapter include ultracentrifugation methods for EV fractionation, chromatographic approaches for analysis of EV lipids, microscopy techniques for analysis of both intracellular and extracellular vesicular compartments, interaction of EVs with host cells, and physical chemical analysis of EVs by dynamic light scattering.

3.2596 Functional exosome-mimic for delivery of siRNA to cancer: in vitro and in vivo evaluation
Yang, Z., Xie, J., Zhu, J., kang, C., Chiang, C., Wang, X., Wang, X., Kuang,k T., Chen, F., Chen, Z., Zhang, A., Yu, B., Lee, R.J., Teng, L.and Lee, L.J.
*J. Controlled Release, **243**, 160-171 (2016)*

Exosomes, the smallest subgroup of extracellular vesicles, have been recognized as extracellular organelles that contain genetic and proteomic information for long distance intercellular communication. Exosome-based drug delivery is currently a subject of intensive research. Here, we report a novel strategy to produce nanoscale exosome-mimics (EMs) in sufficient quantity for gene delivery in cancer both *in vitro* and *in vivo*. Size-controllable EMs were generated at a high yield by serial extrusion of non-tumorigenic epithelial MCF-10A cells through filters with different pore sizes. siRNA was then encapsulated into the EMs by electroporation. Biosafety and uptake efficiency of the EMs were evaluated both *in vitro* and *in vivo*. The mechanism underlying their cellular endocytosis was also studied.

3.2597 Outer Membrane Vesicle Production Facilitates LPS Remodeling and Outer Membrane Maintenance in Salmonella during Environmental Transitions
Bonnington, K. and Kuehn, M.J.

*mBio, **7(5)**, e01532-16 (2016)*

The ability of Gram-negative bacteria to carefully modulate outer membrane (OM) composition is essential to their survival. However, the asymmetric and heterogeneous structure of the Gram-negative OM poses unique challenges to the cell's successful adaption to rapid environmental transitions. Although mechanisms to recycle and degrade OM phospholipid material exist, there is no known mechanism by which to remove unfavorable lipopolysaccharide (LPS) glycoforms, except slow dilution through cell growth. As all Gram-negative bacteria constitutively shed OM vesicles (OMVs), we propose that cells may utilize OMV formation as a way to selectively remove environmentally disadvantageous LPS species. We examined the native kinetics of OM composition during physiologically relevant environmental changes in *Salmonella enterica*, a well-characterized model system for activation of PhoP/Q and PmrA/B two-component systems (TCSs). In response to acidic pH, toxic metals, antimicrobial peptides, and lack of divalent cations, these TCSs modify the LPS lipid A and core, lengthen the O antigen, and upregulate specific OM proteins. An environmental change to PhoP/Q- and PmrA/B-activating conditions simultaneously induced the addition of modified species of LPS to the OM, downregulation of previously dominant species of LPS, greater OMV production, and increased OMV diameter. Comparison of the relative abundance of lipid A species present in the OM and the newly budded OMVs following two sets of rapid environmental shifts revealed the retention of lipid A species with modified phosphate moieties in the OM concomitant with the selective loss of palmitoylated species via vesiculation following exposure to moderately acidic environmental conditions.

3.2598 Generation, Quantification, and Tracing of Metabolically Labeled Fluorescent Exosomes
Coscia, C., Parolini, I., Sanchez, M., Biffoni, M., Boussadia, Z., Zanetti, C., Fiani, M.L. and Sargiacomo, M.
*Methods in Mol. Biol., **1448**, 217-235 (2016)*

Over the last 10 years, the constant progression in exosome (Exo)-related studies highlighted the importance of these cell-derived nano-sized vesicles in cell biology and pathophysiology. Functional studies on Exo uptake and intracellular trafficking require accurate quantification to assess sufficient and/or necessary Exo particles quantum able to elicit measurable effects on target cells. We used commercially available BODIPY® fatty acid analogues to label a primary melanoma cell line (Me501) that highly and spontaneously secrete nanovesicles. Upon addition to cell culture, BODIPY fatty acids are rapidly incorporated into major phospholipid classes ultimately producing fluorescent Exo as direct result of biogenesis. Our metabolic labeling protocol produced bright fluorescent Exo that can be examined and quantified with conventional non-customized flow cytometry (FC) instruments by exploiting their fluorescent emission rather than light-scattering detection. Furthermore, our methodology permits the measurement of single Exo-associated fluorescence transfer to cells making quantitative the correlation between Exo uptake and activation of cellular processes. Thus the protocol presented here appears as an appropriate tool to who wants to investigate mechanisms of Exo functions in that it allows for direct and rapid characterization and quantification of fluorescent Exo number, intensity, size, and eventually evaluation of their kinetic of uptake/secretion in target cells.

3.2599 Mechanism of Fibronectin Binding to Human Trabecular Meshwork Exosomes and Its Modulation by Dexamethasone
Dismuke, W.M., Klingeborn, M. and Stamer, W.D.
*PloS One, **11(10)**, e0165326 (2016)*

Exosomes are emerging as important mediators of cell-matrix interactions by means of specific adhesion proteins. Changes in the tissue-specific exosomal protein expression may underlie pathological conditions whereby extracellular matrix turnover and homeostasis is disrupted. Ocular hypertension due to extracellular matrix accumulation in the trabecular meshwork is a hallmark of glucocorticoid-induced glaucoma. In the trabecular meshwork, exosomal fibronectin mediates cell matrix interactions at cellular structures called "invadosomes". Trabecular meshwork cells use invadosomes to turn over their surrounding matrix and maintain passageways for flow of aqueous humor. In this study, we observed that human trabecular meshwork explants treated with dexamethasone released exosomes with significantly reduced amounts of fibronectin bound per exosome. Further, we found that exosome-fibronectin binding is heparan sulfate-dependent, consistent with our observation that trabecular meshwork exosomes are enriched in the heparin/heparan sulfate binding annexins A2 and A6. In this way, dexamethasone-treated explants released exosomes with a significant reduction in annexin A2 and A6 per exosome. Interestingly, we did not detect exosomal matrix metalloproteinases, but we identified abundant dipeptidyl peptidase 4, a

serine protease whose activity was reduced on exosomes isolated from dexamethasone-treated explants. Together, our findings demonstrate mechanistically how corticosteroid-induced alterations in exosomal adhesion cargo and properties can account for the pathological matrix accumulation seen in many glaucoma patients.

3.2600 **Plasma Membrane Microdomains Are Essential for Rac1-RbohB/H-Mediated Immunity in Rice**
Nagano, M., Ishikawa, T., Fujiwara, M., Fukao, Y., Kawano, Y., Kawai-Yamada, M. and Shimamoto, K.
*Plant Cell, **28(8)**, 1966-1983 (2016)*

Numerous plant defense-related proteins are thought to congregate in plasma membrane microdomains, which consist mainly of sphingolipids and sterols. However, the extent to which microdomains contribute to defense responses in plants is unclear. To elucidate the relationship between microdomains and innate immunity in rice (*Oryza sativa*), we established lines in which the levels of sphingolipids containing 2-hydroxy fatty acids were decreased by knocking down two genes encoding fatty acid 2-hydroxylases (*FAH1* and *FAH2*) and demonstrated that microdomains were less abundant in these lines. By testing these lines in a pathogen infection assay, we revealed that microdomains play an important role in the resistance to rice blast fungus infection. To illuminate the mechanism by which microdomains regulate immunity, we evaluated changes in protein composition, revealing that microdomains are required for the dynamics of the Rac/ROP small GTPase Rac1 and respiratory burst oxidase homologs (Rbohs) in response to chitin elicitor. Furthermore, FAHs are essential for the production of reactive oxygen species (ROS) after chitin treatment. Together with the observation that RbohB, a defense-related NADPH oxidase that interacts with Rac1, is localized in microdomains, our data indicate that microdomains are required for chitin-induced immunity through ROS signaling mediated by the Rac1-RbohB pathway.

3.2601 **Hepatitis B virus inhibits insulin receptor signaling and impairs liver regeneration via intracellular retention of the insulin receptor**
Barthel, S.R., Medvedev, R., Heinrich, T., Büchner, S.M., Kettern, N. and Hildt, E.
*Cell. Mol. Life Sci., **73(21)**, 4121-4140 (2016)*

Hepatitis B virus (HBV) causes severe liver disease but the underlying mechanisms are incompletely understood. During chronic HBV infection, the liver is recurrently injured by immune cells in the quest for viral elimination. To compensate tissue injury, liver regeneration represents a vital process which requires proliferative insulin receptor signaling. This study aims to investigate the impact of HBV on liver regeneration and hepatic insulin receptor signaling. After carbon tetrachloride-induced liver injury, liver regeneration is delayed in HBV transgenic mice. These mice show diminished hepatocyte proliferation and increased expression of fibrosis markers. This is in accordance with a reduced activation of the insulin receptor although HBV induces expression of the insulin receptor via activation of NF-E2-related factor 2. This leads to increased intracellular amounts of insulin receptor in HBV expressing hepatocytes. However, intracellular retention of the receptor simultaneously reduces the amount of functional insulin receptors on the cell surface and thereby attenuates insulin binding in vitro and in vivo. Intracellular retention of the insulin receptor is caused by elevated amounts of α-taxilin, a free syntaxin binding protein, in HBV expressing hepatocytes preventing proper targeting of the insulin receptor to the cell surface. Consequently, functional analyses of insulin responsiveness revealed that HBV expressing hepatocytes are less sensitive to insulin stimulation leading to delayed liver regeneration. This study describes a novel pathomechanism that uncouples HBV expressing hepatocytes from proliferative signals and thereby impedes compensatory liver regeneration after liver injury.

3.2602 **Endogenous macrophage migration inhibitory factor reduces the accumulation and toxicity of misfolded SOD1 in a mouse model of ALS**
Leyton-Jaimes, M.F., Benaim, C., Abu-hamad, S., Kahn, J., Guetta, A., Bucala, R. and Israelson, A.
*PNAS, **113(36)**, 10198-10293 (2016)*

Mutations in superoxide dismutase (SOD1) cause amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease characterized by the loss of upper and lower motor neurons in the brain and spinal cord. It has been suggested that the toxicity of mutant SOD1 results from its misfolding and accumulation on the cytoplasmic faces of intracellular organelles, including the mitochondria and endoplasmic reticulum (ER) of ALS-affected tissues. Recently, macrophage migration inhibitory factor (MIF) was shown to directly inhibit the accumulation of misfolded SOD1 and its binding to intracellular membranes, but the role of endogenous MIF in modulating SOD1 misfolding in vivo remains unknown. To elucidate this role, we bred MIF-deficient mice with $SOD1^{G85R}$ mice, which express a dismutase-

inactive mutant of SOD1 and are considered a model of familial ALS. We found that the accumulation of misfolded SOD1, its association with mitochondrial and ER membranes, and the levels of sedimentable insoluble SOD1 aggregates were significantly higher in the spinal cords of $SOD1^{G85R}$-$MIF^{-/-}$ mice than in their $SOD1^{G85R}$-$MIF^{+/+}$ littermates. Moreover, increasing MIF expression in neuronal cultures inhibited the accumulation of misfolded SOD1 and rescued from mutant SOD1-induced cell death. In contrast, the complete elimination of endogenous MIF accelerated disease onset and late disease progression and shortened the lifespan of the $SOD1^{G85R}$ mutant mice. These findings indicate that MIF plays a significant role in the folding and misfolding of SOD1 in vivo, and they have implications for the potential therapeutic role of up-regulating MIF within the nervous system to modulate the selective accumulation of misfolded SOD1.

3.2603 Polycomb repressive complex 2 (PRC2) silences genes responsible for neurodegeneration
Von Schimmelmann, M., Feinberg, P.A., Sullivan, J.M., Ku, S.M., Badimon, A., Duff, M.K., Wang, Z., Lachmann, A., Dewell, S., Ma'ayan, A., Han, M-H., Tarakhovsky, A. and Schaefer, A.
Nature Neurosci., ***19(10)***, *1321-1330 (2016)*

Normal brain function depends on the interaction between highly specialized neurons that operate within anatomically and functionally distinct brain regions. Neuronal specification is driven by transcriptional programs that are established during early neuronal development and remain in place in the adult brain. The fidelity of neuronal specification depends on the robustness of the transcriptional program that supports the neuron type-specific gene expression patterns. Here we show that polycomb repressive complex 2 (PRC2), which supports neuron specification during differentiation, contributes to the suppression of a transcriptional program that is detrimental to adult neuron function and survival. We show that PRC2 deficiency in striatal neurons leads to the de-repression of selected, predominantly bivalent PRC2 target genes that are dominated by self-regulating transcription factors normally suppressed in these neurons. The transcriptional changes in PRC2-deficient neurons lead to progressive and fatal neurodegeneration in mice. Our results point to a key role of PRC2 in protecting neurons against degeneration.

3.2604 Protein kinase C controls lysosome biogenesis independently of mTORC1
Li, Y. et al
Nature Cell Biol., ***18(10)***, *1065-1077 (2016)*

Lysosomes respond to environmental cues by controlling their own biogenesis, but the underlying mechanisms are poorly understood. Here we describe a protein kinase C (PKC)-dependent and mTORC1-independent mechanism for regulating lysosome biogenesis, which provides insights into previously reported effects of PKC on lysosomes. By identifying lysosome-inducing compounds we show that PKC couples activation of the TFEB transcription factor with inactivation of the ZKSCAN3 transcriptional repressor through two parallel signalling cascades. Activated PKC inactivates GSK3β, leading to reduced phosphorylation, nuclear translocation and activation of TFEB, while PKC activates JNK and p38 MAPK, which phosphorylate ZKSCAN3, leading to its inactivation by translocation out of the nucleus. PKC activation may therefore mediate lysosomal adaptation to many extracellular cues. PKC activators facilitate clearance of aggregated proteins and lipid droplets in cell models and ameliorate amyloid β plaque formation in APP/PS1 mouse brains. Thus, PKC activators are viable treatment options for lysosome-related disorders.

3.2605 The lysosomal membrane protein SCAV-3 maintains lysosome integrity and adult longevity
Li, Y., Chen, B., Zou, W., Wang, X., Wu, Y., Zhao, D., Sun, Y., Liu, Y., Chen, L., Miao, L., Yang, C. and Wang, X.
J. Cell Biol., ***215(2)***, *167-185 (2016)*

Lysosomes degrade macromolecules and recycle metabolites as well as being involved in diverse processes that regulate cellular homeostasis. The lysosome is limited by a single phospholipid bilayer that forms a barrier to separate the potent luminal hydrolases from other cellular constituents, thus protecting the latter from unwanted degradation. The mechanisms that maintain lysosomal membrane integrity remain unknown. Here, we identified SCAV-3, the *Caenorhabditis elegans* homologue of human LIMP-2, as a key regulator of lysosome integrity, motility, and dynamics. Loss of *scav-3* caused rupture of lysosome membranes and significantly shortened lifespan. Both of these phenotypes were suppressed by reinforced expression of LMP-1 or LMP-2, the *C. elegans* LAMPs, indicating that longevity requires maintenance of lysosome integrity. Remarkably, reduction in insulin/insulin-like growth factor 1 (IGF-1)

signaling suppressed lysosomal damage and extended the lifespan in *scav-3(lf)* animals in a DAF-16–dependent manner. Our data reveal that SCAV-3 is essential for preserving lysosomal membrane stability and that modulation of lysosome integrity by the insulin/IGF-1 signaling pathway affects longevity.

3.2606 Dual-Wavelength Surface Plasmon Resonance for Determining the Size and Concentration of Sub-Populations of Extracellular Vesicles

Rupert, D., Shelke, G.V., Emilsson, G., Claudio, V., Block, S., Lässer, C., Dahlin, A., Lötvall, J.O., Bally, M., Zhdanov, V.P. and Höök, F.
Anal. Chem., ***88(20)***, *9980-9988 (2016)*

Accurate concentration determination of subpopulations of extracellular vesicles (EVs), such as exosomes, is of importance both in the context of understanding their fundamental biological role and of potentially using them as disease biomarkers. In principle, this can be achieved by measuring the rate of diffusion-limited mass uptake to a sensor surface modified with a receptor designed to only bind the subpopulation of interest. However, a significant error is introduced if the targeted EV subpopulation has a size, and thus hydrodynamic diffusion coefficient, that differs from the mean size and diffusion coefficient of the whole EV population and/or if the EVs become deformed upon binding to the surface. We here demonstrate a new approach to determine the mean size (or effective film thickness) of bound nanoparticles, in general, and EV subpopulation carrying a marker of interest, in particular. The method is based on operating surface plasmon resonance simultaneously at two wavelengths with different sensing depths and using the ratio of the corresponding responses to extract the particle size on the surface. By estimating in this way the degree of deformation of adsorbed EVs, we markedly improved their bulk concentration determination and showed that EVs carrying the exosomal marker CD63 correspond to not more than around 10% of the EV sample.

3.2607 Aminochrome induces dopaminergic neuronal dysfunction: a new animal model for Parkinson's disease

Herrera, A., Munoz, P., paris, I., Diaz-Veliz, G., Mora, S., Inzunza, J., Hultenby, K., Caardenas, C., Jana, F., Raisman-Vozari, R., Gysling, K., Abarca, J., Steinbusch, H.W. and Segura-Aguilar, J.
Cell. Mol. Life Sci., ***73(18)***, *3583-3597 (2016)*

l-Dopa continues to be the gold drug in Parkinson's disease (PD) treatment from 1967. The failure to translate successful results from preclinical to clinical studies can be explained by the use of preclinical models which do not reflect what happens in the disease since these induce a rapid and extensive degeneration; for example, MPTP induces a severe Parkinsonism in only 3 days in humans contrasting with the slow degeneration and progression of PD. This study presents a new anatomy and develops preclinical model based on aminochrome which induces a slow and progressive dysfunction of dopaminergic neurons. The unilateral injection of aminochrome into rat striatum resulted in (1) contralateral rotation when the animals are stimulated with apomorphine; (2) absence of significant loss of tyrosine hydroxylase-positive neuronal elements both in substantia nigra and striatum; (3) cell shrinkage; (4) significant reduction of dopamine release; (5) significant increase in GABA release; (6) significant decrease in the number of monoaminergic presynaptic vesicles; (7) significant increase of dopamine concentration inside of monoaminergic vesicles; (8) significant increase of damaged mitochondria; (9) significant decrease of ATP level in the striatum (10) significant decrease in basal and maximal mitochondrial respiration. These results suggest that aminochrome induces dysfunction of dopaminergic neurons where the contralateral behavior can be explained by aminochrome-induced ATP decrease required both for anterograde transport of synaptic vesicles and dopamine release. Aminochrome could be implemented as a new model neurotoxin to study Parkinson's disease.

3.2608 Sortilin regulates sorting and secretion of Sonic hedgehog

Campbell, C., Beug, S., Nickerson, P.E.B., peng, J., Mazerolle, C., Bassett, E.A., Ringuette, R., Jama, F.A., Morales, C., Christ, A. and Wallace, A.
J. Cell Sci., ***129(20)***, *3832-3844 (2016)*

Sonic Hedgehog (Shh) is a secreted morphogen that is an essential regulator of patterning and growth. The Shh full-length protein undergoes autocleavage in the endoplasmic reticulum to generate the biologically active N-terminal fragment (ShhN), which is destined for secretion. We identified sortilin (Sort1), a member of the VPS10P-domain receptor family, as a new Shh trafficking receptor. We demonstrate that Sort–Shh interact by performing coimmunoprecipitation and proximity ligation assays in transfected cells and that they colocalize at the Golgi. Sort1 overexpression causes re-distribution of ShhN and, to a lesser

extent, of full-length Shh to the Golgi and reduces Shh secretion. We show loss of Sort1 can partially rescue Hedgehog-associated patterning defects in a mouse model that is deficient in Shh processing, and we show that Sort1 levels negatively regulate anterograde Shh transport in axons *in vitro* and Hedgehog-dependent axon–glial interactions *in vivo*. Taken together, we conclude that Shh and Sort1 can interact at the level of the Golgi and that Sort1 directs Shh away from the pathways that promote its secretion

3.2609 A Portrait of the Human Organelle Proteome In Space and Time during Cytomegalovirus Infection
Beltran, P.M., Mathias, R.A. and Cristea, I.M.
Cell Systems, ***3(4)***, *361-373 (2016)*

The organelles within a eukaryotic host are manipulated by viruses to support successful virus replication and spread of infection, yet the global impact of viral infection on host organelles is poorly understood. Integrating microscopy, subcellular fractionation, mass spectrometry, and functional analyses, we conducted a cell-wide study of organelles in primary fibroblasts throughout the time course of human cytomegalovirus (HCMV) infection. We used label-free and isobaric-labeling proteomics to characterize nearly 4,000 host and 100 viral proteins, then classified their specific subcellular locations over time using machine learning. We observed a global reorganization of proteins across the secretory pathway, plasma membrane, and mitochondria, including reorganization and processing of lysosomal proteins into distinct subpopulations and translocations of individual proteins between organelles at specific time points. We also demonstrate that MYO18A, an unconventional myosin that translocates from the plasma membrane to the viral assembly complex, is necessary for efficient HCMV replication. This study provides a comprehensive resource for understanding host and virus biology during HCMV pathogenesis.

3.2610 Commercial Dairy Cow Milk microRNAs Resist Digestion under Simulated Gastrointestinal Tract Conditions
Benmoussa, A., Lee, C.H.C., laffont, B., Savard, P., laugier, J., Boilard, E., Gilbert, C., Fliss, I. and provost, P.
J. Nutr., ***146***, *2206-2215 (2016)*

Background: MicroRNAs are small, gene-regulatory noncoding RNA species present in large amounts in milk, where they seem to be protected against degradative conditions, presumably because of their association with exosomes.
Objective: We monitored the relative stability of commercial dairy cow milk microRNAs during digestion and examined their associations with extracellular vesicles (EVs).
Methods: We used a computer-controlled, in vitro, gastrointestinal model TNO intestinal model-1 (TIM-1) and analyzed, by quantitative polymerase chain reaction, the concentration of 2 microRNAs within gastrointestinal tract compartments at different points in time. EVs within TIM-1 digested and nondigested samples were studied by immunoblotting, dynamic light scattering, quantitative polymerase chain reaction, and density measurements.
Results: A large quantity of dairy milk *Bos taurus* microRNA-223 (bta-miR-223) and bta-miR-125b ($\sim 10^9$–10^{10} copies/300 mL milk) withstood digestion under simulated gastrointestinal tract conditions, with the stomach causing the most important decrease in microRNA amounts. A large quantity of these 2 microRNAs ($\sim 10^8$–10^9 copies/300 mL milk) was detected in the upper small intestine compartments, which supports their potential bioaccessibility. A protocol optimized for the enrichment of dairy milk exosomes yielded a 100,000 × *g* pellet fraction that was positive for the exosomal markers tumor susceptibility gene-101 (TSG101), apoptosis-linked gene 2–interacting protein X (ALIX), and heat shock protein 70 (HSP70) and containing bta-miR-223 and bta-miR-125b. This approach, based on successive ultracentrifugation steps, also revealed the existence of $ALIX^-$, $HSP70^{-/low}$, and $TSG101^{-/low}$ EVs larger than exosomes and 2–6 times more enriched in bta-miR-223 and bta-miR-125b ($P < 0.05$).
Conclusions: Our findings indicate that commercial dairy cow milk contains numerous microRNAs that can resist digestion and are associated mostly with $ALIX^-$, $HSP70^{-/low}$, and $TSG101^{-/low}$ EVs. Our results support the existence of interspecies transfer of microRNAs mediated by milk consumption and challenge our current view of exosomes as the sole carriers of milk-derived microRNAs.

3.2611 Sphingosine Kinase 1 Cooperates with Autophagy to Maintain Endocytic Membrane Trafficking
Yuong, M.M., Takahashi, Y., Fox, T.E., Yun, J.K., kester, M. and Wang, H-G.
Cell Reports, ***17***, *1532-1545 (2016)*

Sphingosine kinase 1 (Sphk1) associates with early endocytic membranes during endocytosis; however, the role of sphingosine or sphingosine-1-phosphate as the critical metabolite in endocytic trafficking has

not been established. Here, we demonstrate that the recruitment of Sphk1 to sphingosine-enriched endocytic vesicles and the generation of sphingosine-1-phosphate facilitate membrane trafficking along the endosomal pathway. Exogenous sphingosine and sphingosine-based Sphk1 inhibitors induce the Sphk1-dependent fusion of endosomal membranes to accumulate enlarged late endosomes and amphisomes enriched in sphingolipids. Interestingly, Sphk1 also appears to facilitate endosomal fusion independent of its catalytic activity, given that catalytically inactive $Sphk1^{G82D}$ is recruited to endocytic membranes by sphingosine or sphingosine-based Sphk1 inhibitor and promotes membrane fusion. Furthermore, we reveal that the clearance of enlarged endosomes is dependent on the activity of ceramide synthase, lysosomal biogenesis, and the restoration of autophagic flux. Collectively, these studies uncover intersecting roles for Sphk1, sphingosine, and autophagic machinery in endocytic membrane trafficking.

3.2612 **Characterization of Heparan Sulfate Proteoglycan-positive Recycling Endosomes Isolated from Glioma Cells**
Podyma-Inoue, K.A., Morikawi, T., Rajapakshe, A.A., Terasawa, K. and hara-Yokoyama, M.
*Cancer Genomics Proteomics, **13**, 443-452 (2016)*

Background: Heparan sulfate proteoglycans (HSPGs)-dependent endocytic events have been involved in glioma progression. Thus, comprehensive understanding of the intracellular trafficking complexes formed in presence of HSPGs would be important for development of glioma treatments. Materials and Methods: Subcellular fractionation was used to separate vesicles containing HSPGs from the rat C6 glioma cell line. Isolated HSPG-positive vesicles were further characterized with liquid chromatography-mass spectrometry. Results: The HSPG-positive vesicular fractions, distinct from plasma membrane-derived material, were enriched in endocytic marker, Rab11. Proteomic analysis identified more than two hundred proteins to be associated with vesicular membrane, among them, over eighty were related to endosomal uptake, recycling or vesicular transport. Conclusion: Part of HSPGs in glioma cells is internalized through clathrin-dependent endocytosis and undergo recycling. The development of compounds regulating HSPG-mediated trafficking will likely enable design of effective glioma treatment.

3.2613 **FREE**

3.2614 **Trehalose prevents aggregation of exosomes and cryodamage**
Bosch, S., de Beaurepaire, L., Allard, M., Mosser, M., Heichette, C., Chretien, D., Jegou, D. and Bach, J-M.
*Scientific Reports, **6**:36263 (2016)*

Exosomes are important mediators in intercellular communication. Released by many cell types, they transport proteins, lipids, and nucleic acids to distant recipient cells and contribute to important physiopathological processes. Standard current exosome isolation methods based on differential centrifugation protocols tend to induce aggregation of particles in highly concentrated suspensions and freezing of exosomes can induce damage and inconsistent biological activity. Trehalose is a natural, non-toxic sugar widely used as a protein stabilizer and cryoprotectant by the food and drug industry. Here we report that addition of 25 mM trehalose to pancreatic beta-cell exosome-like vesicle isolation and storage buffer narrows the particle size distribution and increases the number of individual particles per microgram of protein. Repeated freeze-thaw cycles induce an increase in particle concentration and in the width of the size distribution for exosome-like vesicles stored in PBS, but not in PBS 25 mM trehalose. No signs of lysis or incomplete vesicles were observed by cryo-electron tomography in PBS and trehalose samples. In macrophage immune assays, beta-cell extracellular vesicles in trehalose show consistently higher TNF-alpha cytokine secretion stimulation indexes suggesting improved preservation of biological activity. The addition of trehalose might be an attractive means to standardize experiments in the field of exosome research and downstream applications.

3.2615 **Detailed Analysis of Protein Topology of Extracellular Vesicles–Evidence of Unconventional Membrane Protein Orientation**
Cvjetkovic, A., Jang, S.C., Konecna, B., Höög, J.L., Sihlbom, C., Lässer, C. and Lötvall, J.
*Scientific Reports, **6**:36338 (2016)*

Extracellular vesicles (EVs) are important mediators of intercellular communication that change the recipient cell by shuttling lipids, RNA, or protein cargo between cells. Here, we investigate the topology of

the protein cargo found in EVs, as this topology can fundamentally influence the biological effects of EVs. A multiple proteomics approach, combining proteinase treatment and biotin tagging, shows that many proteins of cytosolic origin are localized on the surface of EVs. A detailed analysis of the EV proteome at the peptide level revealed that a number of EV membrane proteins are present in a topologically reversed orientation compared to what is annotated. Two examples of such proteins, SCAMP3 and STX4, were confirmed to have a reversed topology. This reversed typology was determined using flow cytometry and fluorescent microscopy with antibodies directed toward their cytoplasmic epitopes. These results describe a novel workflow to define the EV proteome and the orientation of each protein, including membrane protein topology. These data are fundamentally important to understanding the EV proteome and required to fully explain EV biogenesis as well as biological function in recipient cells.

3.2616 Characterization of exosomal release in bovine endometrial intercaruncular stromal cells

Koh, Y.Q., Peiris, H.N., Vaswani, K., Reed, S., Rice, G.E., Salomon, C. and Mitchell, M.D:
*Reprod.Biol. Endocrinol., **14**:478 (2016)*

Cell-to-cell communication between the blastocyst and endometrium is critical for implantation. In recent years, evidence has emerged from studies in humans and several other animal species that exosomes are secreted from the endometrium and trophoblast cells and may play an important role in cell-to-cell communication maternal-fetal interface during early pregnancy. Exosomes are stable extracellular lipid bilayer vesicles that encapsulate proteins, miRNAs, and mRNAs, with the ability to deliver their cargo to near and distant sites, altering cellular function(s). Furthermore, the exosomal cargo can be altered in response to environmental cues (e.g. hypoxia). The current study aims to develop an in vitro system to evaluate maternal-embryo interactions via exosomes (and exosomal cargo) produced by bovine endometrial stromal cells (ICAR) using hypoxia as a known stimulus associated with the release of exosomes and alterations to biological responses (e.g. cell proliferation).
Methods
ICAR cells cultured under 8 % O2 or 1 % O2 for 48 h and changes in cell function (i.e. migration, proliferation and apoptosis) were evaluated. Exosome release was determined following the isolation (via differential centrifugation) and characterization of exosomes from ICAR cell-conditioned media. Exosomal proteomic content was evaluated by mass spectrometry.
Results
Under hypoxic conditions (i.e. 1 % O2), ICAR cell migration and proliferation was decreased (~20 and ~32 %, respectively) and apoptotic protein caspase-3 activation was increased (~1.6 fold). Hypoxia increased exosome number by ~3.6 fold compared with culture at 8 % O2. Mass spectrometry analysis identified 128 proteins unique to exosomes of ICAR cultured at 1 % O2 compared with only 46 proteins unique to those of ICAR cultured at 8 % O2. Differential production of proteins associated with specific biological processes and molecular functions were identified, most notably ADAM10, pantetheinase and kininogen 2.
Conclusions
In summary, we have shown that a stimulus such as hypoxia can alter both the cellular function and exosome release of ICAR cells. Alterations to exosome release and exosomal content in response to stimuli may play a crucial role in maternal-fetal crosstalk and could also affect placental development.

3.2617 Membrane Interactions of the Mason-Pfizer Monkey Virus Matrix Protein and Its Budding Deficient Mutants

Kroupa,, T., langerova, H., Dolezal, M., Prchal, J., Spiwok, V., Hunter, E., Rumlova, M., Hrabal, R. and Ruml, T.
*J. Mol. Biol., **428**, 4708-4722 (2016)*

Matrix proteins (MAs) play a key role in the transport of retroviral proteins inside infected cells and in the interaction with cellular membranes. In most retroviruses, retroviral MAs are N-terminally myristoylated. This modification serves as a membrane targeting signal and also as an anchor for membrane interaction. The aim of this work was to characterize the interactions anchoring retroviral MA at the plasma membrane of infected cell. To address this issue, we compared the structures and membrane affinity of the Mason-Pfizer monkey virus (M-PMV) wild-type MA with its two budding deficient double mutants, that is, T41I/T78I and Y28F/Y67F. The structures of the mutants were determined using solution NMR spectroscopy, and their interactions with water-soluble phospholipids were studied. Water-soluble phospholipids are widely used models for studying membrane interactions by solution NMR spectroscopy. However, this approach might lead to artificial results due to unnatural hydrophobic interactions. Therefore, we used a new approach based on the measurement of the loss of the ^{1}H NMR signal intensity of the

protein sample induced by the addition of the liposomes containing phospholipids with naturally long fatty acids. HIV-1 MA was used as a positive control because its ability to interact with liposomes has already been described. We found that in contrast to HIV-1, the M-PMV MA interacted with the liposomes differently and much weaker. In our *invivo* experiments, the M-PMV MA did not co-localize with lipid rafts. Therefore, we concluded that M-PMV might adopt a different membrane binding mechanism than HIV-1.

3.2618 Outer membrane vesicles containing signalling molecules and active hydrolytic enzymes released by a coral pathogen Vibrio shilonii AK1
Li, J., Azam, F. and Zhang, S.
*Environmental Microbiol., **18(11)**, 3850-3866 (2016)*

Production and release of outer-membrane vesicles (OMVs) is known in many bacteria including human pathogens. To date, OMV release has not been reported in coral-associated bacteria. We discovered that *Vibrio shilonii* AK1, a well-studied coral pathogen, produces OMVs in culture. Transmission electron microscopy showed that *V. shilonii* cultures release two types of vesicles, with a single membrane or two membranes, as well as vesicle chain-like morphotype in purified vesicle fraction. No significant difference was observed in the amount of OMVs produced by cultures grown at 20°C or 30°C. OMV proteomic analysis, never before done in a coral isolate, showed that a large number of low abundance proteins were exclusively detected in OMVs released by 20°C cultures. Further, the OMVs purified from AK1 cultures grown at both 20°C and 30°C carry N-acylhomoserine lactone quorum sensing signals, as well as alkaline phosphatase, lipase and chitinase activities. Our results show that *V. shilonii* OMVs are conduits of signalling molecules, active enzymes and other proteins to its environment. These findings suggest important ecophysiological roles of OMVs in coral reef environment. We discuss the importance of OMV release for *V. shilonii* fitness and propose several hypotheses as well as a conceptual model.

3.2619 N-linked glycosylation at Asn152 on CD147 affects protein folding and stability: promoting tumour metastasis in hepatocellular carcinoma
Li, J-H., Huang, W., Lin, P., Wu, B., Fu, Z-G., Shen, H-M., Jing, L., Liu, Z-Y., Zhou, Y., Meng, Y., Xu, B-Q., Chen, Z-N. and Jiang, J.L.
*Scientific Reports, **6**:35210 (2016)*

Cluster of differentiation 147 (CD147), also known as extracellular matrix metalloproteinase inducer, is a transmembrane glycoprotein that mediates oncogenic processes partly through N-glycosylation modifications. N-glycosylation has been demonstrated to be instrumental for the regulation of CD147 function during malignant transformation. However, the role that site-specific glycosylation of CD147 plays in its defective function in hepatocellular carcinomacells needs to be determined. Here, we demonstrate that the modification of N-glycosylation at Asn152 on CD147 strongly promotes hepatocellular carcinoma (HCC) invasion and migration. After the removal of N-glycans at Asn152, CD147 was more susceptible to degradation by ER-localized ubiquitin ligase-mediated endoplasmic reticulum-associated degradation (ERAD). Furthermore, N-linked glycans at Asn152 were required for CD147 to acquire and maintain proper folding in the ER. Moreover, N-linked glycans at Asn152 functioned as a recognition motif that was directly mediated by the CNX quality control system. Two phases in the retention-based ER chaperones system drove ER-localized CD147 trafficking to degradation. Deletion of N-linked glycosylation at Asn152 on CD147 significantly suppressed in situ tumour metastasis. These data could potentially shed light on the molecular regulation of CD147 through glycosylation and provide a valuable means of developing drugs that target N-glycans at Asn152 on CD147.

3.2620 Cerebral vascular amyloid seeds drive amyloid β-protein fibril assembly with a distinct anti-parallel structure
Xu, F., Fu, Z., Dass, S., Kotarba, A.E., Davis, J., Smith, S.O. and Van Nostrand, W.E.
*Nature Communications, **7**:13527 (2016)*

Cerebrovascular accumulation of amyloid β-protein (Aβ), a condition known as cerebral amyloid angiopathy (CAA), is a common pathological feature of patients with Alzheimer's disease. Familial Aβ mutations, such as Dutch-E22Q and Iowa-D23N, can cause severe cerebrovascular accumulation of amyloid that serves as a potent driver of vascular cognitive impairment and dementia. The distinctive features of vascular amyloid that underlie its unique pathological properties remain unknown. Here, we use transgenic mouse models producing CAA mutants (Tg-SwDI) or overproducing human wild-type Aβ (Tg2576) to demonstrate that CAA-mutant vascular amyloid influences wild-type Aβ deposition in brain.

We also show isolated microvascular amyloid seeds from Tg-SwDI mice drive assembly of human wild-type Aβ into distinct anti-parallel β-sheet fibrils. These findings indicate that cerebrovascular amyloid can serve as an effective scaffold to promote rapid assembly and strong deposition of Aβ into a unique structure that likely contributes to its distinctive pathology.

3.2621 The Warburg Effect Mediator Pyruvate Kinase M2 Expression and Regulation in the Retina
Rajala, R.V.S., Rajala, A., Kooker, C., Wang, Y. and Anderson, R.E.
*Scientific Reports, **6**:37727 (2016)*

The tumor form of pyruvate kinase M2 (PKM2) undergoes tyrosine phosphorylation and gives rise to the Warburg effect. The Warburg effect defines a pro-oncogenic metabolism switch such that cancer cells take up more glucose than normal tissue and favor incomplete oxidation of glucose, even in the presence of oxygen. Retinal photoreceptors are highly metabolic and their energy consumption is equivalent to that of a multiplying tumor cell. In the present study, we found that PKM2 is the predominant isoform in both rod- and cone-dominant retina, and that it undergoes a light-dependent tyrosine phosphorylation. We also discovered that PKM2 phosphorylation is signaled through photobleaching of rhodopsin. Our findings suggest that phosphoinositide 3-kinase activation promotes PKM2 phosphorylation. Light and tyrosine phosphorylation appear to regulate PKM2 to provide a metabolic advantage to photoreceptor cells, thereby promoting cell survival.

3.2622 Outer membrane vesicles derived from Salmonella Typhimurium mutants with truncated LPS induce cross-protective immune responses against infection of Salmonella enterica serovars in the mouse model
Liu, Q., Liu, Q., Yi, J., Liang, K., Liu, T., Roland, K.L., Jiang, Y. and Kong, Q.
*Int. J. Med. Microbiol., **306**, 697-706 (2016)*

Salmonella enterica cause diarrheal and systemic diseases and are of considerable concern worldwide. Vaccines that are cross-protective against multiple serovars could provide effective control of *Salmonella*-mediated diseases. Bacteria-derived outer membrane vesicles (OMVs) are highly immunogenic and are capable of eliciting protective immune responses. Alterations in lipopolysaccharide (LPS) length can result in outer membrane remodeling and composition of outer membrane proteins (OMPs) changing. In this study, we investigated the impact of truncated LPS on both the production and immunogenicity of *Salmonella* OMVs, including the ability of OMVs to elicit cross-protection against challenge by heterologous *Salmonella* strains. We found that mutations in *waaJ* and *rfbP* enhanced vesiculation, while mutations in *waaC*, *waaF* and *waaG* inhibited this process. Animal experiments indicated that OMVs from *waaC*, *rfaH* and *rfbP* mutants induced stronger serum immune responses compared to OMVs from the parent strain, while all elicited protective responses against the wild-type *S.* Typhimurium challenge. Furthermore, intranasal or intraperitoneal immunization with OMVs derived from the *waaC* and *rfbP* mutants elicited significantly higher cross-reactive IgG responses and provided enhanced cross-protection against *S.* Choleraesuis and *S.* Enteritidis challenge than the wild-type OMVs. These results indicate that truncated-LPS OMVs are capable of conferring cross protection against multiple serotypes of *Salmonella* infection.

3.2623 Extracellular vesicles for drug delivery
Vader, P., Mol., E.A., Pasterkamp, G. and Schiffelers, R.M.
*Adv. Drug Delivery Reviews, **106**, 148-156 (2016)*

Extracellular vesicles (EVs) are cell-derived membrane vesicles, and represent an endogenous mechanism for intercellular communication. Since the discovery that EVs are capable of functionally transferring biological information, the potential use of EVs as drug delivery vehicles has gained considerable scientific interest. EVs may have multiple advantages over currently available drug delivery vehicles, such as their ability to overcome natural barriers, their intrinsic cell targeting properties, and stability in the circulation. However, therapeutic applications of EVs as drug delivery systems have been limited due to a lack of methods for scalable EV isolation and efficient drug loading. Furthermore, in order to achieve targeted drug delivery, their intrinsic cell targeting properties should be tuned through EV engineering. Here, we review and discuss recent progress and remaining challenges in the development of EVs as drug delivery vehicles.

3.2624 Therapeutic and diagnostic applications of extracellular vesicles
Stremersch, S., De Smedt, S.C. and Raemdonck, K.

*J. Controlled Release, **244**, 167-183 (2016)*

During the past two decades, extracellular vesicles (EVs) have been identified as important mediators of intercellular communication, enabling the functional transfer of bioactive molecules from one cell to another. Consequently, it is becoming increasingly clear that these vesicles are involved in many (patho)physiological processes, providing opportunities for therapeutic applications. Moreover, it is known that the molecular composition of EVs reflects the physiological status of the producing cell and tissue, rationalizing their exploitation as biomarkers in various diseases. In this review the composition, biogenesis and diversity of EVs is discussed in a therapeutic and diagnostic context. We describe emerging therapeutic applications, including the use of EVs as drug delivery vehicles and as cell-free vaccines, and reflect on future challenges for clinical translation. Finally, we discuss the use of EVs as a biomarker source and highlight recent studies and clinical successes.

3.2625 A novel Rab10-EHBP1-EHD2 complex essential for the autophagic engulfment of lipid droplets
Li, Z., Schulze, R.J., Weller, S.G., Krueger, E.W., Schott, M.B., Zhang, X., Casey, C.A., Liu, J., Stöckli, J., James, D.E. and McNiven, M.A.
*Sci. Adv., **2**, e1601470 (2016)*

The autophagic digestion of lipid droplets (LDs) through lipophagy is an essential process by which most cells catabolize lipids as an energy source. However, the cellularmachinery used for the envelopment of LDs during autophagy is poorly understood.We report a novel function for a small Rab guanosine triphosphatase (GTPase) in the recruitment of adaptors required for the engulfment of LDs by the growing autophagosome. In hepatocytes stimulated to undergo autophagy, Rab10 activity is amplified significantly, concomitant with its increased recruitment to nascent autophagic membranes at the LD surface. Disruption of Rab10 function by small interfering RNA knockdown or expression of a GTPase-defective variant leads to LD accumulation. Finally, Rab10 activation during autophagy is essential for LC3 recruitment to the autophagosome and stimulates its increased association with the adaptor protein EHBP1 (EH domain binding protein 1) and the membrane-deforming adenosine triphosphatase EHD2 (EH domain containing 2) that, together, are essential in driving the activated “engulfment” of LDs during lipophagy in hepatocytes.

3.2626 Extracellular Vesicles Move Toward Use in Clinical Laboratories
Strotman, L.N. and Linder, M.W.
*Clin. Lab. Med., **36(3)**, 587-602 (2016)*

Extracellular vesicles (EVs) are membranous particles found in a variety of biofluids that encapsulate molecular information from the cell, from which they originate.

This rich source of information that is easily obtained can then be mined to find diagnostic biomarkers.

The biological function of EVs are still evolving, but include, intercellular communication, coagulation, inflammation, immune response modulations, waste management and disease progression.

Interest in EVs has expanded but much still needs to be done for EVs to reach their full potential as a source material in laboratory developed tests for personalized medicine, especially in oncology.

3.2627 Calcium- and Nitric Oxide-Dependent Nuclear Accumulation of Cytosolic Glyceraldehyde-3-Phosphate Dehydrogenase in Response to Long Chain Bases in Tobacco BY-2 Cells
Testard, A., Da Silva, D., Ormancey, M., Pichereaux, C., Pouzet, C., Jauneau, A., Grat, S., Robe, E., Briere, C., Cotelle, V., Mazars, C. and Thuleau, P.
*Plant Cell Physiol., **57(10)**, 2221-2231 (2016)*

Sphinganine or dihydrosphingosine (d18:0, DHS), one of the most abundant free sphingoid long chain bases (LCBs) in plants, is known to induce a calcium-dependent programmed cell death (PCD) in plants. In addition, in tobacco BY-2 cells, it has been shown that DHS triggers a rapid production of H_2O_2 and nitric oxide (NO). Recently, in analogy to what is known in the animal field, plant cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC), a ubiquitous enzyme involved in glycolysis, has been suggested to fulfill other functions associated with its oxidative post-translational modifications such as S-nitrosylation on cysteine residues. In particular, in mammals, stress signals inducing NO production promote S-nitrosylation of GAPC and its subsequent translocation into the nucleus where the protein

participates in the establishment of apoptosis. In the present study, we investigated the behavior of GAPC in tobacco BY-2 cells treated with DHS. We found that upon DHS treatment, an S-nitrosylated form of GAPC accumulated in the nucleus. This accumulation was dependent on NO production. Two genes encoding GAPCs, namely Nt(BY-2)GAPC1 and Nt(BY-2)GAPC2, were cloned. Transient overexpression of Nt(BY-2)GAPC–green fluorescent protein (GFP) chimeric constructs indicated that both proteins localized in the cytoplasm as well as in the nucleus. Mutating into serine the two cysteine residues thought to be S-nitrosylated in response to DHS did not modify the localization of the proteins, suggesting that S-nitrosylation of GAPCs was probably not necessary for their nuclear relocalization. Interestingly, using Förster resonance energy transfer experiments, we showed that Nt(BY-2)GAPCs interact with nucleic acids in the nucleus. When GAPCs were mutated on their cysteine residues, their interaction with nucleic acids was abolished, suggesting a role for GAPCs in the protection of nucleic acids against oxidative stress.

3.2628 Extracellular vesicles in diagnosis and therapy of kidney diseases
Zhang, W., Zhou, X., Xhang, H., Yao, Q., Liu, Y. and Dong, Z.
Am. J. Physiol. Renal Physiol., ***311(5)***, *F844-F851 (2016)*

Extracellular vesicles (EV) are endogenously produced, membrane-bound vesicles that contain various molecules. Depending on their size and origins, EVs are classified into apoptotic bodies, microvesicles, and exosomes. A fundamental function of EVs is to mediate intercellular communication. In kidneys, recent research has begun to suggest a role of EVs, especially exosomes, in cell-cell communication by transferring proteins, mRNAs, and microRNAs to recipient cells as nanovectors. EVs may mediate the cross talk between various cell types within kidneys for the maintenance of tissue homeostasis. They may also mediate the cross talk between kidneys and other organs under physiological and pathological conditions. EVs have been implicated in the pathogenesis of both acute kidney injury and chronic kidney diseases, including renal fibrosis, end-stage renal disease, glomerular diseases, and diabetic nephropathy. The release of EVs with specific molecular contents into urine and plasma may be useful biomarkers for kidney disease. In addition, EVs produced by cultured cells may have therapeutic effects for these diseases. However, the role of EVs in kidney diseases is largely unclear, and the mechanism underlying EV production and secretion remains elusive. In this review, we introduce the basics of EVs and then analyze the present information about the involvement, diagnostic value, and therapeutic potential of EVs in major kidney diseases.

3.2629 Comparative Analysis of Ciliary Membranes and Ectosomes
Long, H., Zhang, F., Xu, N., Liu, G., Diener, D.R., Rosenbaum, J.L. and Huang, K.
Current Biology, ***26(24)***, *3327-3335 (2016)*

Primary and motile cilia/flagella function as cellular antennae, receiving signals from the environment and subsequently activating signaling pathways that are critical for cellular homeostasis and differentiation [1–3]. Recent work with the green alga *Chlamydomonas* and the nematode *C. elegans* demonstrated that ectosomes can be released from the cilium and can mediate the intercellular communication [4–9]. To better understand the function of flagellar ectosomes, we have compared their protein composition to that of the flagellar membrane from which they are derived. Ectosomes released from flagella have a unique protein composition, being enriched in a subset of flagellar membrane proteins, proteases, proteins from the endosomal sorting complex required for transport (ESCRT) [10–12], small GTPases, and ubiquitinated proteins. Live imaging showed that an ESCRT-related protein (PDCD6) was enriched in ectosomes released from flagella during gamete activation. We devised a sensitive and rapid assay to monitor ectosome release using luciferase fused to PDCD6 and a mutated ubiquitin. Ectosome release increased when cells underwent flagellar resorption. Knockdown of two ESCRT-related proteins, PDCD6 and VPS4, attenuated ectosome release during flagellar shortening and shortening was slowed. These data suggest that the ESCRT proteins mediate ectosome release and thereby influence flagellar shortening in *Chlamydomonas.* In addition, the prevalence of receptors such as agglutinin and ubiquitinated proteins in ciliary ectosomes suggests that they are involved in cell signaling and turnover of ciliary proteins.

3.2630 PIKfyve inhibition increases exosome release and induces secretory autophagy
Pettersen Hessvik, N., Øverbye, A., Brech, A., Lyngaas Torgersen, M., Seim jacobsen, I., Sandvig, K. And Llorente, A.
Cell. Mol. Life Sci., ***73(24)***, *4717-4737 (2016)*

Exosomes are vesicles released from cells by fusion of multivesicular bodies (MVBs) with the plasma membrane. This study aimed to investigate whether the phosphoinositide kinase PIKfyve affects this

process. Our results show that in PC-3 cells inhibition of PIKfyve by apilimod or depletion by siRNA increased the secretion of the exosomal fraction. Moreover, quantitative electron microscopy analysis showed that cells treated with apilimod contained more MVBs per cell and more intraluminal vesicles per MVB. Interestingly, mass spectrometry analysis revealed a considerable enrichment of autophagy-related proteins (NBR1, p62, LC3, WIPI2) in exosomal fractions released by apilimod-treated cells, a result that was confirmed by immunoblotting. When the exosome preparations were investigated by electron microscopy a small population of p62-labelled electron dense structures was observed together with CD63-containing exosomes. The p62-positive structures were found in less dense fractions than exosomes in density gradients. Inside the cells, p62 and CD63 were found in the same MVB-like organelles. Finally, both the degradation of EGF and long-lived proteins were shown to be reduced by apilimod. In conclusion, inhibition of PIKfyve increases secretion of exosomes and induces secretory autophagy, showing that these pathways are closely linked. We suggest this is due to impaired fusion of lysosomes with both MVBs and autophagosomes, and possibly increased fusion of MVBs with autophagosomes, and that the cells respond by secreting the content of these organelles to maintain cellular homeostasis.

3.2631 **Silver nanoparticle–protein interactions in intact rainbow trout gill cells**
Yue, Y., Behra, R., Sigg, L., Suter, M.J-F., Pillai, S. and Schirmer, K.
*Environ. Sci. Nano, **3**, 1174-1185 (2016)*

Upon contact with biota, nanoparticles can bind to proteins, which coat the nanoparticles and form a nanoparticle-protein corona. Knowledge of corona proteins is therefore important for a mechanistic understanding of how nanoparticles interact with biomolecules in cells. Here we present the first study to reveal the identity of corona proteins from silver nanoparticle (AgNPs)-exposed living vertebrate cells. The cells are from a rainbow trout (***Oncorhynchus mykiss***) gill cell line, RTgill-W1, representing the interface between the aquatic environment and one of its model species. Subcellular fractionation allowed AgNP-protein corona complexes to be recovered from intact subcellular compartments and proteins lysed from the AgNPs to be detected by mass spectrometry. The identified proteins mark the trail of AgNPs processing in the cells like a forensic fingerprint: the cells take up the AgNPs ***via*** endocytic processes and store the particles in endosomal/lysosomal compartments. Moreover, stress response proteins were recovered in the AgNPs protein corona. In this way, we established a list of AgNPs susceptible proteins which can be investigated further in targeted nanoparticle–protein interaction. As a proof of principle, we demonstrate that Na^+/K^+-ATPase, identified from the corona and a known key protein in ion regulation in gill cells, is inhibited in its activity by AgNPs, confirming previously published ***in vivo*** experiments. The developed methodology is broadly applicable to other nanoparticles and cell types, representing a valuable tool for mechanistic nanoparticle–cell interaction studies, ranging from environmental and human risk assessment to biomedicine. In this way, our research also contributes to safer particle design.

3.2632 **Pex9p is a new yeast peroxisomal import receptor for PTS1-containing proteins**
Effelsberg, D., Cruz-Zaragoza, L.D., Schliebs, W. and Erdmann, R.
*J. Cell Sci., **129**, 4057-4066 (2016)*

Peroxisomal proteins carrying a type 1 peroxisomal targeting signal (PTS1) are recognized by the well-conserved cycling import receptor Pex5p. The yeast *YMR018W* gene encodes a Pex5p paralog and newly identified peroxin that is involved in peroxisomal import of a subset of matrix proteins. The new peroxin was designated Pex9p, and it interacts with the docking protein Pex14p and a subclass of PTS1-containing peroxisomal matrix enzymes. Unlike Pex5p, Pex9p is not expressed in glucose- or ethanol-grown cells, but it is strongly induced by oleate. Under these conditions, Pex9p acts as a cytosolic and membrane-bound peroxisome import receptor for both malate synthase isoenzymes, Mls1p and Mls2p. The inducible Pex9p-dependent import pathway provides a mechanism for the oleate-inducible peroxisomal targeting of malate synthases. The existence of two distinct PTS1 receptors, in addition to two PTS2-dependent import routes, contributes to the adaptive metabolic capacity of peroxisomes in response to environmental changes and underlines the role of peroxisomes as multi-purpose organelles. The identification of different import routes into peroxisomes contributes to the molecular understanding of how regulated protein targeting can alter the function of organelles according to cellular needs

3.2633 **N-3 vs. n-6 fatty acids differentially influence calcium signalling and adhesion of inflammatory activated monocytes: impact of lipid rafts**
Schaefer, M.B. et al
*Inflamm. Res., **65(11)**, 881-894 (2016)*

Background
Anti-inflammatory n-3 fatty acids (FA) like docosahexaenoic acid (DHA) opposed to the pro-inflammatory n-6 FA arachidonic acid (AA) might modulate lipid rafts within the cell membrane by differential incorporation. In inflammation, monocyte adhesion to endothelial cells is a crucial step mediated by intracellular calcium changes. We investigated whether lipid rafts mediate FA-induced modulation of adhesion and intracellular calcium.
Methods
In isolated human monocytes and monocytic U937 cells we measured adhesion to human umbilical vein endothelial cells (HUVEC) using a parallel flow chamber and a static assay, adhesion molecules by FACScan, and intracellular calcium by fluorescence. Monocyte lipid rafts were isolated by ultracentrifugation and submitted to gas chromatography for FA analysis.
Results
Pre-incubation with AA or DHA resulted in a predominant incorporation of the respective FA into raft compared to non-raft fraction. DHA as compared to AA significantly reduced monocyte adhesion and calcium release after stimulation with TNF-α while expression of adhesion molecules remained unchanged. Pre-treatment with a calcium chelator abolished the effect of FA on calcium and adhesion. Disruption of lipid rafts prevented FA-induced modulations.
Conclusion
Incorporation of FA into lipid rafts seem to be crucial for modulation of adhesion under inflammatory conditions.

3.2634 Mast Cell Degranulation Is Accompanied by the Release of a Selective Subset of Extracellular Vesicles That Contain Mast Cell–Specific Proteases

Groot Kormelink, T., Arkesteijn, G.J.A., van de Lest, C.H.A., Geerts, J.C., Goerdayal, S.S., Altelaar, M.A.F., Redegeld, F.A., Nolte-t' Hoen, E.N.M. and Wauben, M.H.M.
J. Immunol., ***197(8)***, *3382-3392 (2016)*

Mast cells (MC) are well known for their effector role in allergic disorders; moreover, they are associated with diverse modulatory effects in innate and adaptive immunity. It is largely unclear how MC exert these modulating functions. In this article, we show that IgE-mediated MC degranulation leads to a rapid release of high quantities of extracellular vesicles (EV), comparable to the release of preformed mediators. EV are submicron structures composed of lipid bilayers, proteins, and nucleic acids that are released by cells in a regulated fashion and are involved in intercellular communication. Primary murine mucosal-type MC and connective tissue–type MC released phenotypically different EV populations depending on the stimulus they received. Although unstimulated MC constitutively released $CD9^+$ EV, degranulation was accompanied by the release of $CD63^+$ EV, which correlated with release of the soluble mediator β-hexosaminidase. This $CD63^+$ EV subset was smaller and exhibited a higher buoyant density and distinct phospholipid composition compared with $CD9^+$ EV. Marked differences were observed for phosphatidylinositol, phosphatidic acid, and bis(monoacylglycero)phosphate species. Strikingly, proteomic analysis of $CD63^+$ EV from connective tissue–type MC unveiled an abundance of MC-specific proteases. With regard to carboxypeptidase A3, it was confirmed that the enzyme was EV associated and biologically active. Our data demonstrate that, depending on their activation status, MC release distinct EV subsets that differ in composition and protease activity and are indicative of differential immunological functions. Concerning the strategic tissue distribution of MC and the presence of degranulated MC in various (allergic) disorders, MC-derived EV should be considered potentially important immune regulators.

3.2635 Exosome-associated release, uptake, and neurotoxicity of HIV-1 Tat protein

Rahimian, P. and He, J.J.
J. Neurovirol., ***22(6)***, *774-788 (2016)*

HIV-1 Tat is an indispensible transactivator for HIV gene transcription and replication. It has been shown to exit cells as a free protein and enter neighboring cells or interact with surface receptors of neighboring cells to regulate gene expression and cell function. In this study, we report, for the first time, exosome-associated Tat release and uptake. Using a HIV-1 LTR-driven luciferase reporter-based cell assay and Western blotting or in combination with exosome inhibitor, OptiPrep gradient fractionation, and exosome depletion, we demonstrated significant presence of HIV-1 Tat in exosomes derived from Tat-expressing primary astrocytes, Tat-transfected U373.MG and 293T, and HIV-infected MT4. We further showed that exosome-associated Tat from Tat-expressing astrocytes was capable of causing neurite shortening and neuron death, further supporting that this new form of extracellular Tat is biologically active. Lastly, we constructed a Tat mutant deleted of its basic domain and determined the role of the basic domain in Tat

trafficking into exosomes. Basic domain-deleted Tat exhibited no apparent effects on Tat trafficking into exosomes, while maintained its dominant-negative function in Tat-mediated LTR transactivation. Taken together, these results show a significant fraction of Tat is secreted and present in the form of exosomes and may contribute to the stability of extracellular Tat and broaden the spectrum of its target cells.

3.2636 Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation
Abramowicz, A., Widlak, P and Pietrowska, M.
*Molecular Biosystems, **12**, 1407-1419 (2016)*

The re-discovery of exosomes as intercellular messengers with high potential for diagnostic and therapeutic utility has led to them becoming a popular topic of research in recent years. One of the essential research areas in this field is the characterization of exosomal cargo, which includes numerous non-randomly packed proteins and nucleic acids. Unexpectedly, a very challenging aspect of exploration of extracellular vesicles has turned out to be their effective and selective isolation. The plurality of developed protocols leads to qualitative and quantitative variability in terms of the obtained exosomes, which significantly affects the results of downstream analyses and makes them difficult to compare, reproduce and interpret between research groups. Currently, there is a general consensus among the exosome-oriented community concerning the urgent need for the optimization and standardization of methods employed for the purification of these vesicles. Hence, we review here several strategies for exosome preparation including ultracentrifugation, chemical precipitation, affinity capturing and filtration techniques. The advantages and disadvantages of different approaches are discussed with special emphasis being placed on their adequacy for proteomics applications, which are particularly sensitive to sample quality. We conclude that certain methods, exemplified by ultracentrifugation combined with iodixanol density gradient centrifugation or gel filtration, although labor-intensive, provide superior quality exosome preparations suitable for reliable analysis by mass spectrometry.

3.2637 Translocon component Sec62 acts in endoplasmic reticulum turnover during stress recovery
Fumagelli, F. et al
*Nature Cell Biol., **18(11)**, 1173-1184 (2016)*

The endoplasmic reticulum (ER) is a site of protein biogenesis in eukaryotic cells. Perturbing ER homeostasis activates stress programs collectively called the unfolded protein response (UPR). The UPR enhances production of ER-resident chaperones and enzymes to reduce the burden of misfolded proteins. On resolution of ER stress, ill-defined, selective autophagic programs remove excess ER components. Here we identify Sec62, a constituent of the translocon complex regulating protein import in the mammalian ER, as an ER-resident autophagy receptor. Sec62 intervenes during recovery from ER stress to selectively deliver ER components to the autolysosomal system for clearance in a series of events that we name recovER-phagy. Sec62 contains a conserved LC3-interacting region in the C-terminal cytosolic domain that is required for its function in recovER-phagy, but is dispensable for its function in the protein translocation machinery. Our results identify Sec62 as a critical molecular component in maintenance and recovery of ER homeostasis.

3.2638 L1-associated genomic regions are deleted in somatic cells of the healthy human brain
Erwin, J.A., paquola, A., Singer, T., Gallina, I., Novotny, M., Quayle, C., Bedrosian, T.A., Alves, F.I.A., Butcher, C.R., Herdy, J.R., Sarkar, A., Lasken, R.S., Muotri, A.R. and Gage F.H.
*Nature Neurosci., **19(12)**, 1583-1591 (2016)*

healthy human brain is a mosaic of varied genomes. Long interspersed element-1 (LINE-1 or L1) retrotransposition is known to create mosaicism by inserting L1 sequences into new locations of somatic cell genomes. Using a machine learning-based, single-cell sequencing approach, we discovered that somatic L1-associated variants (SLAVs) are composed of two classes: L1 retrotransposition insertions and retrotransposition-independent L1-associated variants. We demonstrate that a subset of SLAVs comprises somatic deletions generated by L1 endonuclease cutting activity. Retrotransposition-independent rearrangements in inherited L1s resulted in the deletion of proximal genomic regions. These rearrangements were resolved by microhomology-mediated repair, which suggests that L1-associated genomic regions are hotspots for somatic copy number variants in the brain and therefore a heritable genetic contributor to somatic mosaicism. We demonstrate that SLAVs are present in crucial neural genes, such as *DLG2* (also called *PSD93*), and affect 44–63% of cells of the cells in the healthy brain.

3.2639 p62- and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome
Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y.T.
*PNAS, **113(47)**, E7490-E7499 (2016)*

The ubiquitin-proteasome system and autophagy are the two main proteolytic systems involved in, among other functions, the maintenance of cell integrity by eliminating misfolded and damaged proteins and organelles. Both systems remove their targets after their conjugation with ubiquitin. An interesting, yet incompletely understood problem relates to the fate of the components of the two systems. Here we provide evidence that amino acid starvation enhances polyubiquitination on specific sites of the proteasome, a modification essential for its targeting to the autophagic machinery. The uptake of the ubiquitinated proteasome is mediated by its interaction with the ubiquitin-associated domain of p62/SQSTM1, a process that also requires interaction with LC3. Importantly, deletion of the PB1 domain of p62, which is important for the targeting of ubiquitinated substrates to the proteasome, has no effect on stress-induced autophagy of this proteolytic machinery, suggesting that the domain of p62 that binds to the proteasome determines the function of p62 in either targeting substrates to the proteasome or targeting the proteasome to autophagy.

3.2640 Fas/CD95 prevents autoimmunity independently of lipid raft localization and efficient apoptosis induction
Cruz, A.C. et al
*Nature Communications, **7**:13895 (2016)*

Mutations affecting the apoptosis-inducing function of the Fas/CD95 TNF-family receptor result in autoimmune and lymphoproliferative disease. However, Fas can also costimulate T-cell activation and promote tumour cell growth and metastasis. Palmitoylation at a membrane proximal cysteine residue enables Fas to localize to lipid raft microdomains and induce apoptosis in cell lines. Here, we show that a palmitoylation-defective Fas C194V mutant is defective in inducing apoptosis in primary mouse T cells, B cells and dendritic cells, while retaining the ability to enhance naive T-cell differentiation. Despite inability to efficiently induce cell death, the Fas C194V receptor prevents the lymphoaccumulation and autoimmunity that develops in Fas-deficient mice. These findings indicate that induction of apoptosis through Fas is dependent on receptor palmitoylation in primary immune cells, and Fas may prevent autoimmunity by mechanisms other than inducing apoptosis.

3.2641 Glucocorticoid Cell Priming Enhances Transfection Outcomes in Adult Human Mesenchymal Stem Cells
Kelly, A.M., Plautz, S.A., Zempleni, J. and Pannier, A.K.
*Molecular Therapy, **24(2)**, 331-341 (2018)*

Human mesenchymal stem cells (hMSCs) are one of the most widely researched stem cell types with broad applications from basic research to therapeutics, the majority of which require introduction of exogenous DNA. However, safety and scalability issues hinder viral delivery, while poor efficiency hinders nonviral gene delivery, particularly to hMSCs. Here, we present the use of a pharmacologic agent (glucocorticoid) to overcome barriers to hMSC DNA transfer to enhance transfection using three common nonviral vectors. Glucocorticoid priming significantly enhances transfection in hMSCs, demonstrated by a 3-fold increase in efficiency, 4–15-fold increase in transgene expression, and prolonged transgene expression when compared to transfection without glucocorticoids. These effects are dependent on glucocorticoid receptor binding and caused in part by maintenance of normal metabolic function and increased cellular (5-fold) and nuclear (6–10-fold) DNA uptake over hMSCs transfected without glucocorticoids. Results were consistent across five human donors and in cells up to passage five. Glucocorticoid cell priming is a simple and effective technique to significantly enhance nonviral transfection of hMSCs that should enhance their clinical use, accelerate new research, and decrease reliance on early passage cells.

3.2642 Genome-wide analysis of mRNAs associated with mouse peroxisomes
Yarmishyn, A.A., Kremenskøy, M., Batagov, A.O., Preuss, A., Wong, J.H. and Kurochkin, I.V.
*BMC Genomics, **17**, Suppl 17:1028 (2016)*

Background

RNA is often targeted to be localized to the specific subcellular compartments. Specific localization of mRNA is believed to be an important mechanism for targeting their protein products to the locations, where their function is required.

Results

In this study we performed the genome wide transcriptome analysis of peroxisome preparations from the mouse liver using microarrays. We demonstrate that RNA is absent inside peroxisomes, however it is associated at their exterior via the noncovalent contacts with the membrane proteins. We detect enrichment of specific sets of transcripts in two preparations of peroxisomes, purified with different degrees of stringency. Importantly, among these were mRNAs encoding *bona fide* peroxisomal proteins, such as peroxins and peroxisomal matrix enzymes involved in beta-oxidation of fatty acids and bile acid biosynthesis. The top-most enriched mRNA, whose association with peroxisomes we confirm microscopically was *Hmgcs1*, encoding 3-hydroxy-3-methylglutaryl-CoA synthase, a crucial enzyme of cholesterol biosynthesis pathway. We observed significant representation of mRNAs encoding mitochondrial and secreted proteins in the peroxisomal fractions.

Conclusions

This is a pioneer genome-wide study of localization of mRNAs to peroxisomes that provides foundation for more detailed dissection of mechanisms of RNA targeting to subcellular compartments.

3.2643 Lipid Raft Integrity Is Required for Survival of Triple Negative Breast Cancer Cells

Badana, A., Chintala, M., Varikuti, G., Pudi, N., Kumari, S., Kappala, V.R. and Malla, R.R.
*J. Breast Cancer, **19(4)**, 372-384 (2016)*

PURPOSE:

Lipid rafts are cholesterol enriched microdomains that colocalize signaling pathways involved in cell proliferation, metastasis, and angiogenesis. We examined the effect of methyl-β-cyclodextrin (MβCD)-mediated cholesterol extraction on the proliferation, adhesion, invasion, and angiogenesis of triple negative breast cancer (TNBC) cells.

METHODS:

We measured cholesterol and estimated cell toxicity. Detergent resistant membrane (DRM) and non-DRM fractions were separated using the OptiPrep gradient method. Cell cycles stages were analyzed by flow cytometry, apoptosis was assessed using the TdT-mediated dUTP nick end-labeling assay, and metastasis was determined using a Matrigel invasion assay. Neo-vessel pattern and levels of angiogenic modulators were determined using an *in vitro* angiogenesis assay and an angiogenesis array, respectively.

RESULTS:

The present study found that the cholesterol-depleting agent MβCD, efficiently depleted membrane cholesterol and caused concentration dependent (0.1-0.5 mM) cytotoxicity compared to nystatin and filipin III in TNBC cell lines, MDA-MB 231 and MDA-MB 468. A reduced proportion of caveolin-1 found in DRM fractions indicated a cholesterol extraction-induced disruption of lipid raft integrity. MβCD inhibited 52% of MDA-MB 231 cell adhesion on fibronectin and 56% of MDA-MB 468 cell adhesion on vitronectin, while invasiveness of these cells was decreased by 48% and 52% respectively, following MβCD treatment (48 hours). MβCD also caused cell cycle arrest at the G_2M phase and apoptosis in MDA-MB 231 cells (25% and 58% cells, respectively) and in MDA-MB 468 cells (30% and 38% cells, respectively). We found that MβCD treated cells caused a 52% and 58% depletion of neovessel formation in both MDA-MB 231 and MDA-MB 468 cell lines, respectively. This study also demonstrated that MβCD treatment caused a respective 2.6- and 2.5-fold depletion of tyrosine protein kinase receptor (TEK) receptor tyrosine kinase levels in both TNBC cell lines.

CONCLUSION:

MβCD-induced cholesterol removal enhances alterations in lipid raft integrity, which reduces TNBC cell survival.

3.2644 Self-Renewal of Bone Marrow Stem Cells by Nanovesicles Engineered from Embryonic Stem Cells

Jo, W., Jeong, D., Kim, J. and Park, J.
*Adv. Healthcare Mater., **5(24)**, 3148-3156 (2016)*

Extracellular vesicles can enhance cell proliferation by stimulating signal transduction and delivering genetic materials, and thus may have applications in regenerative medicine and other therapeutic applications. The processes employed to isolate extracellular vesicles, however, are complex and achieve low yield. To overcome these obstacles, a large-scale, micropore device for generating extracellular vesicle-mimetic nanovesicles that have characteristics similar to those of extracellular vesicles is fabricated. The nanovesicles are generated through the self-assembly capability of cell membrane fragments in an

aqueous solution. The nanovesicles enhance the proliferation of murine mesenchymal stem cells (MSCs), stimulate the signal pathway related to cell proliferation, and do not influence the characteristics of murine MSCs. Therefore, these nanovesicles could provide stable MSCs for regenerative medicine and other therapeutic applications.

3.2645 Flow cytometry analysis of DNA ploidy levels and protein profiles distinguish between populations of Lumbriculus (Annelida: Clitellata)
Tweeten, K.A. and Morris, S.J.
*Invertebrate Biology, **135(4)**, 385-399 (2016)*

Variations in DNA ploidy have been observed in *Lumbriculus*, a freshwater annelid, as well as in other clitellates. Interpretation and application of experimental results using these animals may be impacted as ploidy levels affect the protein expression, reproductive behavior, and response to stressors. Ploidy is typically determined by chromosome spreads, a time-consuming and inefficient method. We adapted flow cytometry protocols used on vertebrates and plants to determine the ploidy levels in different populations of *Lumbriculus*, including a laboratory strain (Environmental Protection Agency), a commercial strain (Aquatic Foods), and worms collected from natural habitats. To isolate nuclei, worms were homogenized, filtered to remove cell debris, and centrifuged through Optiprep™ density gradients. Nuclei were recovered, treated with RNAse, and stained with propidium iodide. Flow cytometry of the labeled nuclei showed that *Lumbriculus* from natural habitats in Minnesota and Iowa were diploid, with an estimated genome size of 2.7 pg. Populations from natural habitats in California and Oregon were highly polyploid, as were the laboratory and commercial strains. Chromosome spreads verified the high ploidy levels indicated by flow cytometry results, but also suggested that flow cytometry may be underestimating the DNA content levels. Staining of nuclei with diamidino-2-phenylindole indicated that this may be due to high levels of heterochromatin in nuclei from polyploid forms of *Lumbriculus*. To further compare the populations, proteins in worm homogenates were subjected to isoelectrofocusing gel electrophoresis. Distinct protein profiles were seen; one was shared in common by the diploid worms, the other was characteristic of polyploid populations. Diploid worms could also be distinguished from polyploid worms based on differences in hemoglobin linker proteins. The results further support taxonomic classification of the diploid and polyploid forms of *Lumbriculus* as distinct species.

3.2646 Nitric Oxide Interacts with Caveolin-1 to Facilitate Autophagy-Lysosome-Mediated Claudin-5 Degradation in Oxygen-Glucose Deprivation-Treated Endothelial Cells
Liu, J., Weaver, J., Jin, X., Zhang, Y., Xu, J., Liu, Ke.J., Li, W. and Liu, W.
*Mol. Neurobiol., **53(9)**, 5935-5947 (2016)*

Using in vitro oxygen-glucose deprivation (OGD) model, we have previously demonstrated that 2-h OGD induces rapid, caveolin-1-mediated dissociation of claudin-5 from the cellular cytoskeletal framework and quick endothelial barrier disruption. In this study, we further investigated the fate of translocated claudin-5 and the mechanisms by which OGD promotes caveolin-1 translocation. Exposure of bEND3 cells to 4-h OGD, but not 2-h OGD plus 2-h reoxygenation, resulted in claudin-5 degradation. Inhibition of autophagy or the fusion of autophagosome with lysosome, but not proteasome, blocked OGD-induced claudin-5 degradation. Moreover, knockdown of caveolin-1 with siRNA blocked OGD-induced claudin-5 degradation. Western blot analysis showed a transient colocalization of caveolin-1, claudin-5, and LC3B in autolysosome or lipid raft fractions at 2-h OGD. Of note, inhibiting autophagosome and lysosome fusion sustained the colocalization of caveolin-1, claudin-5, and LC3B throughout the 4-h OGD exposure. EPR spin trapping showed increased nitric oxide (NO) generation in 2-h OGD-treated cells, and inhibiting NO with its scavenger C-PTIO or inducible nitric oxide synthase (iNOS) inhibitor 1400W prevented OGD-induced caveolin-1 translocation and claudin-5 degradation. Taken together, our data provide a novel mechanism underlying endothelial barrier disruption under prolonged ischemic conditions, in which NO promotes caveolin-1-mediated delivery of claudin-5 to the autophagosome for autophagy-lysosome-dependent degradation.

3.2647 Vps35-dependent recycling of Trem2 regulates microglial function
Yin, J., Liu, X., He, Q., Zhou, L., Yan, Z.and Zhao, S.
*Traffic, **17(12)**, 1286-1296 (2016)*

Triggering receptor expressed on myeloid cells 2 (Trem2), an immune-modulatory receptor, is preferentially expressed in microglia of central nervous system. Trem2 might be involved in the

development of Alzheimer's disease (AD) through regulating the inflammatory responses and phagocytosis of microglia. However, the intracellular trafficking of Trem2 remains unclear. In this study, we showed that Trem2 in the plasma membrane underwent endocytosis and recycling. Trem2 is internalized in a clathrin-dependent manner and then recycled back to the plasma membrane through vacuolar protein sorting 35 (Vps35), the key component of cargo recognition core of retromer complex, but not Rab11. When Vps35 is knocked down, Trem2 accumulated in the lysosomes but was not degraded. More importantly, Vps35 deficiency leads to excessive lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression and IL-6 production, which can be abolished by Trem2 overexpression. Furthermore, R47H Trem2, an AD-associated mutant, failed to interact with Vps35 and became unstable compared with wild-type Trem2. Our study suggests that Vps35/retromer is responsible for recycling of Trem2 in the regulation of microglial function such as proinflammatory responses, whereas R47H mutation impairs Trem2 trafficking, which might contribute to AD.

3.2648 Methods for the physical characterization and quantification of extracellular vesicles in biological samples

Rupert, D., Claudio, V., L¨sser, C. and Bally, M.
*Biochim. Biophys. Acta, **1861**, 3164-3179 (2017)*

Background
Our body fluids contain a multitude of cell-derived vesicles, secreted by most cell types, commonly referred to as extracellular vesicles. They have attracted considerable attention for their function as intercellular communication vehicles in a broad range of physiological processes and pathological conditions. Extracellular vesicles and especially the smallest type, exosomes, have also generated a lot of excitement in view of their potential as disease biomarkers or as carriers for drug delivery. In this context, state-of-the-art techniques capable of comprehensively characterizing vesicles in biological fluids are urgently needed.
Scope of review
This review presents the arsenal of techniques available for quantification and characterization of physical properties of extracellular vesicles, summarizes their working principles, discusses their advantages and limitations and further illustrates their implementation in extracellular vesicle research.
Major conclusions
The small size and physicochemical heterogeneity of extracellular vesicles make their physical characterization and quantification an extremely challenging task. Currently, structure, size, buoyant density, optical properties and zeta potential have most commonly been studied. The concentration of vesicles in suspension can be expressed in terms of biomolecular or particle content depending on the method at hand. In addition, common quantification methods may either provide a direct quantitative measurement of vesicle concentration or solely allow for relative comparison between samples.
General significance
The combination of complementary methods capable of detecting, characterizing and quantifying extracellular vesicles at a single particle level promises to provide new exciting insights into their modes of action and to reveal the existence of vesicle subpopulations fulfilling key biological tasks.

3.2649 Mitochondrial peroxiredoxins are essential in regulating the relationship between Drosophila immunity and aging

Odnojoz, O., nakatsuka, K., Klichko, V.I., Nguyen, J., Solis, L.C., Ostling, K., Badinloo, M., Orr, W.C. and Radyuk, S.N.
*Biochim. Biophys Acta, **1863**, 68-80 (2017)*

Previously, we have shown that flies under-expressing the two mitochondrial peroxiredoxins (Prxs), dPrx3 and dPrx5, display increases in tissue-specific apoptosis and dramatically shortened life span, associated with a redox crisis, manifested as changes in GSH:GSSG and accumulation of protein mixed disulfides. To identify specific pathways responsible for the observed biological effects, we performed a transcriptome analysis. Functional clustering revealed a prominent group enriched for immunity-related genes, including a considerable number of NF-kB-dependent antimicrobial peptides (AMP) that are up-regulated in the Prx double mutant. Using qRT-PCR analysis we determined that the age-dependent changes in AMP levels in mutant flies were similar to those observed in controls when scaled to percentage of life span. To further clarify the role of Prx-dependent mitochondrial signaling, we expressed different forms of dPrx5, which unlike the uniquely mitochondrial dPrx3 is found in multiple subcellular compartments, including mitochondrion, nucleus and cytosol. Ectopic expression of dPrx5 in mitochondria but not nucleus or cytosol partially extended longevity under normal or oxidative stress conditions while complete restoration

of life span occurred when all three forms of dPrx5 were expressed from the wild type dPrx5 transgene. When dPrx5 was expressed in mitochondria or in all three compartments, it substantially delayed the development of hyperactive immunity while expression of cytosolic or nuclear forms had no effect on the immune phenotype. The data suggest a critical role of mitochondria in development of chronic activation of the immune response triggered by impaired redox control.

3.2650 Bacterial protoplast-derived nanovesicles for tumor targeted delivery of chemotherapeutics
Kim, O.Y., Dinh, N.T.H., Park, H.T., Choi. S.J., Hong, K. and Gho, Y.S.
Biomaterials, ***113***, *68-79 (2017)*

Increasing incidents of patients diagnosed with cancer have brought massive improvement in the delivery technologies to help patients receiving chemotherapy. However, tumor specific targeting of the chemotherapeutics still remains as a challenge mainly due to the difficulties in the conjugation and manipulation of bio-specific molecules on the surface. Herein, we genetically engineered bacterial protoplast to develop nanovesicles having no toxic outer membrane components that can specifically target and deliver chemotherapeutics to tumor tissues. The bacterial protoplast nanovesicles expressing tumor-targeting moieties on the surface were prepared by serial extrusions through nano-sized membrane filters. The nano-sized vesicular structure of protoplast nanovesicles offers passive targeting to solid tumor site and expression of tumor-targeting moiety enhance tumor-specific uptake *via* receptor-mediated targeting. Chemotherapeutics-loaded in the nanovesicles induce dose-dependent cytotoxicity in tumor cells *in vitro*. Moreover, specific trafficking of drug-loaded nanovesicles to the tumor tissue and efficient prevention of tumor growth in tumor xenografted mice are shown. Importantly, this tumor growth suppression of protoplast nanovesicles has shown to reduce the chemotherapeutics-induced adverse effects after systemic administration to mice. This study offers great potential of protoplast nanovesicles as effective and safe delivery system to optimize and contribute to the development of advanced chemotherapy.

3.2651 Isolation of detergent-resistant membranes (DRMs) from Escherichia coli
GuzmanFlores, J.E., Alvarez, A.F., Poggio, S., Gavilanes-Ruiz, M. and georgellis, D.
Anal. Biochem., ***518***, *1-8 (2017)*

Lipid rafts or membrane microdomains have been proposed to compartmentalize cellular processes by spatially organizing diverse molecules/proteins in eukaryotic cells. Such membrane microdomains were recently reported to also exist in a few bacterial species. In this work, we report the development of a procedure for membrane microdomain isolation from *Escherichia coli* plasma membranes as well as a method to purify the latter. The method here reported could easily be adapted to other gram-negative bacteria, wherein the isolation of this kind of sub-membrane preparation imposes special difficulties. The analysis of isolated membrane microdomains might provide important information on the nature and function of these bacterial structures and permit their comparison with the ones of eukaryotic cells.

3.2652 Dataset of the proteome of purified outer membrane vesicles from the human pathogen Aggregatibacter actinomycetemcomintans
Kieselbach, T. and Oscarsson, J.
Data in Brief, ***10***, *426-431 (2017)*

The Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* is an oral and systemic pathogen, which is linked to aggressive forms of periodontitis and can be associated with endocarditis. The outer membrane vesicles (OMVs) of this species contain effector proteins such as cytolethal distending toxin (CDT) and leukotoxin (LtxA), which they can deliver into human host cells. The OMVs can also activate innate immunity through NOD1- and NOD2-active pathogen-associated molecular patterns. This dataset provides a proteome of highly purified OMVs from *A. actinomycetemcomitans* serotype *e* strain 173. The experimental data do not only include the raw data of the LC-MS/MS analysis of four independent preparations of purified OMVs but also the mass lists of the processed data and the Mascot.dat files from the database searches. In total 501 proteins are identified, of which 151 are detected in at least three of four independent preparations. In addition, this dataset contains the COG definitions and the predicted subcellular locations (PSORTb 3.0) for the entire genome of *A. actinomycetemcomitans* serotype *e* strain SC1083, which is used for the evaluation of the LC-MS/MS data. These data are deposited in ProteomeXchange in the public dataset PXD002509. In addition, a scientific interpretation of this dataset by Kieselbach et al. (2015) [2] is available at http://dx.doi.org/10.1371/journal.pone.0138591.

3.2653 Microfluidic approaches for isolation, detection, and characterization of extracellular vesicles: Current status and future directions
Gholizadeh, S., Draz, M.S., Zarghooni, M., Sanati-Nezhad, A., Ghavami, S., Shafiee, H. and Akbari, M.
*Biosensors and Bioelectronics, **91**, 588-605 (2017)*

Extracellular vesicles (EVs) are cell-derived vesicles present in body fluids that play an essential role in various cellular processes, such as intercellular communication, inflammation, cellular homeostasis, survival, transport, and regeneration. Their isolation and analysis from body fluids have a great clinical potential to provide information on a variety of disease states such as cancer, cardiovascular complications and inflammatory disorders. Despite increasing scientific and clinical interest in this field, there are still no standardized procedures available for the purification, detection, and characterization of EVs. Advances in microfluidics allow for chemical sampling with increasingly high spatial resolution and under precise manipulation down to single molecule level. In this review, our objective is to give a brief overview on the working principle and examples of the isolation and detection methods with the potential to be used for extracellular vesicles. This review will also highlight the integrated on-chip systems for isolation and characterization of EVs.

3.2654 Influence of maternal BMI on the exosomal profile during gestation and their role on maternal systemic inflammation
Elfeky, O., Longo, S., lai, A., Rice, G.E. and Salomom, C.
*Placenta, **50**, 60-69 (2017)*

Recent studies report that 35% of women are either overweight or obese at reproductive age. The placenta continuously releases exosomes across gestation and their concentration is higher in pregnancy complications. While there is considerable interest in elucidating the role of exosomes during gestation, important questions remain to be answered: *i*) Does maternal BMI affect the exosomal profile across gestation? and *ii*) What is the contribution of placenta-derived exosomes to the total number of exosomes present in maternal plasma across gestation? Plasma samples were classified according to the maternal BMI into three groups (n = 15 per group): Lean, overweight, and obese. Total exosomes and specific placenta-derived exosomes were determined by Nanoparticle Tracking Analysis (NanoSight™) using quantum dots coupled with CD63 or PLAP antibodies. The effect of exosomes on cytokine (IL-6, IL-8, IL-10 and TNF-α) release from endothelial cells was established by cytokine array analysis (Bioplex-200). The total number of exosomes present in maternal circulation was strongly correlated with maternal BMI. Between ~12% and ~25% of circulating exosomes in maternal blood are of placental origin during gestation, and the contribution of placental exosomes to the total exosomal population decreases with higher maternal BMI across gestation. Exosomes increase IL-6, IL-8 and TNF-α release from endothelial cells, an effect even higher when exosomes were isolated from obese women compared to lean and overweight. This study established that maternal BMI is a factor that explains a significant component of the variation in the exosomes data. Exosomes may contribute to the maternal systemic inflammation during pregnancy.

3.2655 Isolation of bacterial compartments to track movement of protein synthesis factors
Zhao, H. and martinis, S.A.
*Methods, **113**, 120-126 (2017)*

Aminoacyl-tRNA synthetases (AARSs) comprise an enzyme family that generates and maintains pools of aminoacylated tRNAs, which serve as essential substrates for protein synthesis. Many protein synthesis factors, including tRNA and AARSs also have non-canonical functions. Particularly in mammalian cells, alternate functions of AARSs have been associated with re-distribution in the cell to sites that are removed from translation. Sub-fractionation methods for *E. coli* were designed and optimized to carefully investigate re-localization of bacterial AARSs and tRNA that might aid in conferring alternate activities. Cell fractionation included isolation of the cytoplasm, periplasm, membrane, outer membrane vesicles, and extracellular media. Specific endogenous proteins and RNAs were probed respectively within each fraction via Western blots using antibodies and by Northern blots with primers to unique regions of the nucleic acid.

3.2656 The new obesity-associated protein, neuronal growth regulator 1 (NEGR1), is implicated in Niemann-Pick disease Type C (NPC2)-mediated cholesterol trafficking
Kim, H., Chun, Y., Che, L., Kim, J., Lee, S. and Lee, S.
*Biochem. Biophys. Res. Comm., **482**, 1367-1374 (2017)*

Neuronal growth regulator 1 (NEGR1) is a newly identified raft-associated protein, which has recently been spotlighted as a new locus related to human obesity. Niemann-Pick disease Type C2 (NPC2) protein functions as a key player in the intracellular cholesterol trafficking, and its defect is linked to a fatal human neurodegenerative disease, NPC. In this study, we identified that NEGR1 interacts with NPC2 and increases its protein stability. Ectopically expressed NEGR1 proteins relieved an abnormal cholesterol accumulation in endosomal compartments. Importantly, NEGR1-defective mouse embryonic fibroblast cells exhibit increased cholesterol levels and triglyceride contents. These findings provide the first insight into the role of NEGR1 in intracellular cholesterol homeostasis, possibly explaining the missing link between NEGR1 with human obesity.

3.2657 **MicroRNAs in extracellular vesicles: potential cancer biomarkers**
Kinoshita, T., Yip, K.W., Spence, T. and Liu, F-F.
J. Hum. Genet., ***62(1)***, *67-74 (2017)*

Extracellular vesicles (EV) are small membrane-bound structures that are secreted by various cell types, including tumor cells. Recent studies have shown that EVs are important for cell-to-cell communication, locally and distantly; horizontally transferring DNA, mRNA, microRNA (miRNA), proteins and lipids. In the context of cancer biology, tumor-derived EVs are capable of modifying the microenvironment, promoting tumor progression, immune evasion, angiogenesis and metastasis. miRNAs contained within EVs are functionally associated with cancer progression, metastasis and aggressive tumor phenotypes. These factors, along with their stability in bodily fluids, have led to extensive investigations on the potential role of circulating EV-derived miRNAs as tumor biomarkers. In this review, we summarize the current understanding of circulating EV miRNAs in human cancer, and discuss their clinical utility and challenges in functioning as biomarkers.

3.2658 **Extracellular Vesicles Isolated from the Leaf Apoplast Carry Stress-Response Proteins**
Rutter, B.D. and Innes, R.W.
Plant Physiol., ***173***, *728-741 (2017)*

Exosomes are extracellular vesicles (EVs) that play a central role in intercellular signaling in mammals by transporting proteins and small RNAs. Plants are also known to produce EVs, particularly in response to pathogen infection. The contents of plant EVs have not been analyzed, however, and their function is unknown. Here, we describe a method for purifying EVs from the apoplastic fluids of Arabidopsis (*Arabidopsis thaliana*) leaves. Proteomic analyses of these EVs revealed that they are highly enriched in proteins involved in biotic and abiotic stress responses. Consistent with this finding, EV secretion was enhanced in plants infected with *Pseudomonas syringae* and in response to treatment with salicylic acid. These findings suggest that EVs may represent an important component of plant immune responses.

3.2659 **Bicarbonate-sensing soluble adenylyl cyclase is present in the cell cytoplasm and nucleus of multiple shark tissues**
Roa, J.N. and Tresguerres, M.
Physiol Rep., ***5(2)***, *e13090 (2017)*

The enzyme soluble adenylyl cyclase (sAC) is directly stimulated by bicarbonate (HCO_3^-) to produce the signaling molecule cyclic adenosine monophosphate (cAMP). Because sAC and sAC-related enzymes are found throughout phyla from cyanobacteria to mammals and they regulate cell physiology in response to internal and external changes in pH, CO_2, and HCO_3^-, sAC is deemed an evolutionarily conserved acid-base sensor. Previously, sAC has been reported in dogfish shark and round ray gill cells, where they sense and counteract blood alkalosis by regulating the activity of V-type H^+- ATPase. Here, we report the presence of sAC protein in gill, rectal gland, cornea, intestine, white muscle, and heart of leopard shark *Triakis semifasciata*. Co-expression of sAC with transmembrane adenylyl cyclases supports the presence of cAMP signaling microdomains. Furthermore, immunohistochemistry on tissue sections, and western blots and cAMP-activity assays on nucleus-enriched fractions demonstrate the presence of sAC protein in and around nuclei. These results suggest that sAC modulates multiple physiological processes in shark cells, including nuclear functions.

3.2660 **MicroRNA Profiling of Exosomes**
Daly, M. and O'Driscoll, L.
Methods in Mol. Biol., ***1509***, *37-46 (2017*

Exosomes are nano-sized membrane-bound vesicles released by a range of different cell types. Exosomes have been shown to specifically package certain membrane and cytosolic proteins and nucleic acids. Furthermore, it has been shown that their contents can be transferred to secondary cells, affecting the recipient cells' cellular processes. Exosomes are present in a multitude of body fluids and so represent a novel source of circulating biomarkers. Here, we describe ultracentrifugation methods suitable for the isolation of exosomes from serum and plasma. We also detail transmission electron microscopy, nanoparticle tracking analysis, and immunoblotting methods suitable for the characterization of exosomes.

3.2661 **The *Trypanosoma cruzi* Surface, a Nanoscale Patchwork Quilt**
Mucci, J., Lantos, A.B., Buscaglia, C.A., Leguizamon, M.S. and Campetella, O.
Trends in Parasitol., ***33(2)***, *102-112 (2017)*

The *Trypanosoma cruzi* trypomastigote membrane provides a major protective role against mammalian host-derived defense mechanisms while allowing the parasite to interact with different cell types and trigger pathogenesis. This surface has been historically appreciated as a rather unstructured 'coat', mainly consisting of a continuous layer of glycolipids and heavily *O*-glycosylated mucins, occasionally intercalated with different developmentally regulated molecules displaying adhesive and/or enzymatic properties. Recent findings, however, indicate that the trypomastigote membrane is made up of multiple, densely packed and discrete 10–150 nm lipid-driven domains bearing different protein composition; hence resembling a highly organized 'patchwork quilt' design. Here, we discuss different aspects underlying the biogenesis, assembly, and dynamics of this cutting-edge fashion outfit, as well as its functional implications.

3.2662 **Localization of a Trypanosome Peroxin to the Endoplasmic Reticulum**
Bauer, S.T., McQueeney, K.E., patel, T and Morris, M.T.
J. Eurkaryotic Microbiol., ***64***, *97-105 (2017)*

Trypanosoma brucei is the causative agent of diseases that affect 30,000–50,000 people annually. *Trypanosoma brucei* harbors unique organelles named glycosomes that are essential to parasite survival, which requires growth under fluctuating environmental conditions. The mechanisms that govern the biogenesis of these organelles are poorly understood. Glycosomes are evolutionarily related to peroxisomes, which can proliferate de novo from the endoplasmic reticulum or through the growth and division of existing organelles depending on the organism and environmental conditions. The effect of environment on glycosome biogenesis is unknown. Here, we demonstrate that the glycosome membrane protein, TbPex13.1, is localized to glycosomes when cells are cultured under high glucose conditions and to the endoplasmic reticulum in low glucose conditions. This localization in low glucose was dependent on the presence of a C-terminal tripeptide sequence. Our findings suggest that glycosome biogenesis is influenced by extracellular glucose levels and adds to the growing body of evidence that de novo glycosome biogenesis occurs in trypanosomes. Because the movement of peroxisomal membrane proteins is a hallmark of ER-dependent peroxisome biogenesis, TbPex13.1 may be a useful marker for the study such processes in trypanosomes.

3.2663 **Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy**
Kimura, T., Jia, J., Kumar, S., Choi, S.W., Gu, Y., Mudd, M., Dupont, N., Jiang, S., Peters, R., Farzam, F., Jain, A., Lidke, K.A., Adams, C.M., Johansen, T. and Deretic, V.
EMBO J., ***36***, *42-60 (2017)*

Autophagy is a process delivering cytoplasmic components to lysosomes for degradation. Autophagy may, however, play a role in unconventional secretion of leaderless cytosolic proteins. How secretory autophagy diverges from degradative autophagy remains unclear. Here we show that in response to lysosomal damage, the prototypical cytosolic secretory autophagy cargo IL-1β is recognized by specialized secretory autophagy cargo receptor TRIM16 and that this receptor interacts with the R-SNARE Sec22b to recruit cargo to the LC3-II$^+$ sequestration membranes. Cargo secretion is unaffected by downregulation of syntaxin 17, a SNARE promoting autophagosome–lysosome fusion and cargo degradation. Instead, Sec22b in combination with plasma membrane syntaxin 3 and syntaxin 4 as well as SNAP-23 and SNAP-29 completes cargo secretion. Thus, secretory autophagy utilizes a specialized cytosolic cargo receptor and a dedicated SNARE system. Other unconventionally secreted cargo, such as ferritin, is secreted via the same pathway.

3.2664 Simvastatin promotes NPC1-mediated free cholesterol efflux from lysosomes through CYP7A1/LXRα signalling pathway in oxLDL-loaded macrophages
Xu, X., Zhang, A., Halquist, M.S., Yuan, X., Henderson, S.C., Dewey, W.L., Li, P-L., Li, N. and Zhang, F.
J. Cell. Mol. Med., ***21(2)****, 364-374 (2017)*

Statins, 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors, are the first-line medications prescribed for the prevention and treatment of coronary artery diseases. The efficacy of statins has been attributed not only to their systemic cholesterol-lowering actions but also to their pleiotropic effects that are unrelated to cholesterol reduction. These pleiotropic effects have been increasingly recognized as essential in statins therapy. This study was designed to investigate the pleiotropic actions of simvastatin, one of the most commonly prescribed statins, on macrophage cholesterol homeostasis with a focus on lysosomal free cholesterol egression. With simultaneous nile red and filipin staining, analysis of confocal/multi-photon imaging demonstrated that simvastatin markedly attenuated unesterified (free) cholesterol buildup in macrophages loaded with oxidized low-density lipoprotein but had little effect in reducing the sizes of cholesteryl ester-containing lipid droplets; the reduction in free cholesterol was mainly attributed to decreases in lysosome-compartmentalized cholesterol. Functionally, the egression of free cholesterol from lysosomes attenuated pro-inflammatory cytokine secretion. It was determined that the reduction of lysosomal free cholesterol buildup by simvastatin was due to the up-regulation of Niemann-Pick C1 (NPC1), a lysosomal residing cholesterol transporter. Moreover, the enhanced enzymatic production of 7-hydroxycholesterol by cytochrome P450 7A1 and the subsequent activation of liver X receptor α underscored the up-regulation of NPC1. These findings reveal a novel pleiotropic effect of simvastatin in affecting lysosomal cholesterol efflux in macrophages and the associated significance in the treatment of atherosclerosis.

3.2665 Isolation and characterization of exosomes derived from fertile sheep hydatid cysts
Siles-Lucas, M., Sanchez-Ovejero, C., Gonzalez-Sanchez, M., Gonzalez, E., Falcon-Perez, J., Boufana, B., Fratini, F., Casulli, A. and Manzano-Roman, R.
Vet. Parasitol., ***236****, 23-33 (2017)*

Cystic echinococcosis (CE) is a chronic and complex zoonotic disease. Information on the mechanisms involved in parasite establishment, growth and persistence remain limited. These may be modulated by a crosstalk between extracellular vesicles (EVs). EVs including exosomes and microvesicles are able to carry developmental signaling proteins which coordinate growth and establishment of several parasites. Here, an exosome enriched EV fraction was isolated from hydatid fluid (HF) of fertile sheep cysts. A proteomic analysis of this fraction identified a number of parasite-derived vesicle-membrane associated proteins as well as cytosolic proteinsAdditionally, the exosomal enriched fraction contained proteins of host origin. Specific proteins −antigen B2 and TSPAN14- in the exosomal fraction were further assayed by immunoblot and transmission electron microscopy. To the best of our knowledge, this is the first report on the presence of parasite exosomes in fertile hydatid cyst fluid. Further characterization of the exosome cargo will allow the discovery of new markers for the detection of CE in humans and animals, and the treatment of CE patients, and provide new insights regarding the role of these EVs in the establishment and persistence of hydatid cysts.

3.2666 Endosulfine-alpha inhibits membrane-induced α-synuclein aggregation and protects against α-synuclein neurotoxicity
Ysselstein, D., Dehay, B., Costantino, I.M., McCabe, G.P., Frosch, M.P., George, J.M., Bezard, E. and Rochet, J-C.
Acta Neuropathologica Comm., ***5****:3 (2017)*

Neuropathological and genetic findings suggest that the presynaptic protein α-synuclein (aSyn) is involved in the pathogenesis of synucleinopathy disorders, including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy. Evidence suggests that the self-assembly of aSyn conformers bound to phospholipid membranes in an aggregation-prone state plays a key role in aSyn neurotoxicity. Accordingly, we hypothesized that protein binding partners of lipid-associated aSyn could inhibit the formation of toxic aSyn oligomers at membrane surfaces. To address this hypothesis, we characterized the protein endosulfine-alpha (ENSA), previously shown to interact selectively with membrane-bound aSyn, in terms of its effects on the membrane-induced aggregation and neurotoxicity of two familial aSyn mutants, A30P and G51D. We found that wild-type ENSA, but not the non-aSyn-binding S109E variant, interfered with membrane-induced aSyn self-assembly, aSyn-mediated vesicle disruption and aSyn neurotoxicity. Immunoblotting analyses revealed that ENSA was down-regulated in the brains of

synucleinopathy patients versus non-diseased individuals. Collectively, these results suggest that ENSA can alleviate neurotoxic effects of membrane-bound aSyn via an apparent chaperone-like activity at the membrane surface, and a decrease in ENSA expression may contribute to aSyn neuropathology in synucleinopathy disorders. More generally, our findings suggest that promoting interactions between lipid-bound, amyloidogenic proteins and their binding partners is a viable strategy to alleviate cytotoxicity in a range of protein misfolding disorders.

3.2667 The effects of glomerular and tubular renal progenitors and derived extracellular vesicles on recovery from acute kidney injury

Ranghino, A., Bruno, S., Bussolati, B., Moggio, A., Dimuccio, V., Tapporo, M., Biancone, L., Gontero, P., Frea, B. and Camussi, G.

Stem Cell Res. Ther., ***8****:24 (2017)*

Background
Mesenchymal stromal cells (MSCs) and renal stem/progenitors improve the recovery of acute kidney injury (AKI) mainly through the release of paracrine mediators including the extracellular vesicles (EVs). Several studies have reported the existence of a resident population of MSCs within the glomeruli (Gl-MSCs). However, their contribution towards kidney repair still remains to be elucidated. The aim of the present study was to evaluate whether Gl-MSCs and Gl-MSC-EVs promote the recovery of AKI induced by ischemia-reperfusion injury (IRI) in SCID mice. Moreover, the effects of Gl-MSCs and Gl-MSC-EVs were compared with those of $CD133^+$ progenitor cells isolated from human tubules of the renal cortical tissue (T-$CD133^+$ cells) and their EVs (T-$CD133^+$-EVs).
Methods
IRI was performed in mice by clamping the left renal pedicle for 35 minutes together with a right nephrectomy. Immediately after reperfusion, the animals were divided in different groups to be treated with: Gl-MSCs, T-$CD133^+$ cells, Gl-MSC-EVs, T-$CD133^+$-EVs or vehicle. To assess the role of vesicular RNA, EVs were either isolated by floating to avoid contamination of non-vesicles-associated RNA or treated with a high dose of RNase. Mice were sacrificed 48 hours after surgery.
Results
Gl-MSCs, and Gl-MSC-EVs both ameliorate kidney function and reduce the ischemic damage post IRI by activating tubular epithelial cell proliferation. Furthermore, T-$CD133^+$ cells, but not their EVs, also significantly contributed to the renal recovery after IRI compared to the controls. Floating EVs were effective while RNase-inactivated EVs were ineffective. Analysis of the EV miRnome revealed that Gl-MSC-EVs selectively expressed a group of miRNAs, compared to EVs derived from fibroblasts, which were biologically ineffective in IRI.
Conclusions
In this study, we demonstrate that Gl-MSCs may contribute in the recovery of mice with AKI induced by IRI primarily through the release of EVs.

3.2668 TAX1BP1 Restrains Virus-Induced Apoptosis by Facilitating Itch-Mediated Degradation of the Mitochondrial Adaptor MAVS

Choi, Y.B., Shembade, N., parvatiyar, K., Balachandran, S. and Harhaj, E.W.

Mol. Cell. Biol., ***37(1)****, e00422-16 (2017)*

The host response to RNA virus infection consists of an intrinsic innate immune response and the induction of apoptosis as mechanisms to restrict viral replication. The mitochondrial adaptor molecule MAVS plays critical roles in coordinating both virus-induced type I interferon production and apoptosis; however, the regulation of MAVS-mediated apoptosis is poorly understood. Here, we show that the adaptor protein TAX1BP1 functions as a negative regulator of virus-induced apoptosis. TAX1BP1-deficient cells are highly sensitive to apoptosis in response to infection with the RNA viruses vesicular stomatitis virus and Sendai virus and to transfection with poly(I·C). TAX1BP1 undergoes degradation during RNA virus infection, and loss of TAX1BP1 is associated with apoptotic cell death. TAX1BP1 deficiency augments virus-induced activation of proapoptotic c-Jun N-terminal kinase (JNK) signaling. Virus infection promotes the mitochondrial localization of TAX1BP1 and concomitant interaction with the mitochondrial adaptor MAVS. TAX1BP1 recruits the E3 ligase Itch to MAVS to trigger its ubiquitination and degradation, and loss of TAX1BP1 or Itch results in increased MAVS protein expression. Together, these results indicate that TAX1BP1 functions as an adaptor molecule for Itch to target MAVS during RNA virus infection and thus restrict virus-induced apoptosis.

3.2669 Spatiotemporal Uncoupling of MicroRNA-Mediated Translational Repression and Target RNA Degradation Controls MicroRNP Recycling in Mammalian Cells
Bose, M., Baarman, B., Goswami, A. and Bhattacharyya, S.N.
Mol. Cell. Biol., ***37(4)***, *e00464-16 (2017)*

MicroRNA (miRNA)-mediated repression controls expression of more than half of protein-coding genes in metazoan animals. Translation repression is associated with target mRNA degradation initiated by decapping and deadenylation of the repressed mRNAs. Earlier evidence suggests the endoplasmic reticulum (ER) as the site where microRNPs (miRNPs) interact with their targets before translation repression sets in, but the subcellular location of subsequent degradation of miRNA-repressed messages is largely unidentified. Here, we explore the subcellular distribution of essential components of degradation machineries of miRNA-targeted mRNAs. We have noted that interaction of target mRNAs with AGO2 protein on the ER precedes the relocalization of repressed messages to multivesicular bodies (MVBs). The repressed messages subsequently get deadenylated, lose their interaction with AGO2, and become decapped. Blocking maturation of endosomes to late endosome and MVBs by targeting the endosomal protein HRS uncouples miRNA-mediated translation repression from target RNA degradation. HRS is also targeted by the intracellular parasite Leishmania donovani, which curtails the HRS level in infected cells to prevent uncoupling of mRNA-AGO2 interaction, preventing degradation of translationally repressed messages, and thus stops recycling of miRNPs preengaged in repression.

3.2670 CD63 Regulates Epstein-Barr Virus LMP1 Exosomal Packaging, Enhancement of Vesicle Production, and Noncanonical NF-κB Signaling
Hurwitz, S.N., Nkosi, D., Conlon, M.N., York, S.B., Liu, X., Tremblay, D.C. and Meckes Jr., D.G.
J. Virol., ***91(5)***, *e02251-16 (2017)*

Latent membrane protein 1 (LMP1) is an Epstein-Barr virus (EBV)-encoded oncoprotein that is packaged into small extracellular vesicles (EVs) called exosomes. Trafficking of LMP1 into multivesicular bodies (MVBs) alters the content and function of exosomes. LMP1-modified exosomes enhance the growth, migration, and invasion of malignant cells, demonstrating the capacity to manipulate the tumor microenvironment and enhance the progression of EBV-associated cancers. Despite the growing evidence surrounding the significance of LMP1-modified exosomes in cancer, very little is understood about the mechanisms that orchestrate LMP1 incorporation into these vesicles. Recently, LMP1 was shown to be copurified with CD63, a conserved tetraspanin protein enriched in late endosomal and lysosomal compartments. Here, we demonstrate the importance of CD63 presence for exosomal packaging of LMP1. Nanoparticle tracking analysis and gradient purification revealed an increase in extracellular vesicle secretion and exosomal proteins following LMP1 expression. Immunoisolation of CD63-positive exosomes exhibited accumulation of LMP1 in this vesicle population. Functionally, CRISPR/Cas9 knockout of CD63 resulted in a reduction of LMP1-induced particle secretion. Furthermore, LMP1 packaging was severely impaired in CD63 knockout cells, concomitant with a disruption in the perinuclear localization of LMP1. Importantly, LMP1 trafficking to lipid rafts and activation of NF-κB and PI3K/Akt pathways remained intact following CD63 knockout, while mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and noncanonical NF-κB activation were observed to be increased. These results suggest that CD63 is a critical player in LMP1 exosomal trafficking and LMP1-mediated enhancement of exosome production and may play further roles in limiting downstream LMP1 signaling.

3.2671 Comprehensive proteome profiling of glioblastoma-derived extracellular vesicles identifies markers for more aggressive disease
Mallawaarratchy, D.M., Hallal, S., Russell, B., Ly, L., Wbrahimkhani, S., Wei, H., Christopherson, R.I., Buckland, M.E. and Kaufman, K.L.
J. Neurooncol., ***131***, *233-244 (2017)*

Extracellular vesicles (EVs) play key roles in glioblastoma (GBM) biology and represent novel sources of biomarkers that are detectable in the peripheral circulation. Despite this notionally non-invasive approach to assess GBM tumours in situ, a comprehensive GBM EV protein signature has not been described. Here, EVs secreted by six GBM cell lines were isolated and analysed by quantitative high-resolution mass spectrometry. Overall, 844 proteins were identified in the GBM EV proteome, of which 145 proteins were common to EVs secreted by all cell lines examined; included in the curated EV compendium (Vesiclepedia_559; http://microvesicles.org). Levels of 14 EV proteins significantly correlated with cell invasion (invadopodia production; $r^2 > 0.5$, $p < 0.05$), including several proteins that interact with molecules responsible for regulating invadopodia formation. Invadopodia, actin-rich membrane

protrusions with proteolytic activity, are associated with more aggressive disease and are sites of EV release. Gene levels corresponding to invasion-related EV proteins showed that five genes (annexin A1, actin-related protein 3, integrin-β1, insulin-like growth factor 2 receptor and programmed cell death 6-interacting protein) were significantly higher in GBM tumours compared to normal brain in silico, with common functions relating to actin polymerisation and endosomal sorting. We also show that Cavitron Ultrasonic Surgical Aspirator (CUSA) washings are a novel source of brain tumour-derived EVs, demonstrated by particle tracking analysis, TEM and proteome profiling. Quantitative proteomics corroborated the high levels of proposed invasion-related proteins in EVs enriched from a GBM compared to low-grade astrocytoma tumour. Large-scale clinical follow-up of putative biomarkers, particularly the proposed survival marker annexin A1, is warranted.

3.2672 High-density lipoprotein and apolipoprotein A-I inhibit palmitate-induced translocation of toll-like receptor 4 into lipid rafts and inflammatory cytokines in 3T3-L1 adipocytes
Yamada, H., Umemoto, T., kawano, M., Kawakami, M., Kakei, M., Momomura, S-i., Ishikawa, S-e and Hara, K.
Biochem. Biophys. Res. Comm., ***484***, *403-408 (2017)*

Saturated fatty acids (SFAs) activate toll-like receptor 4 (TLR4) signal transduction in macrophages and are involved in the chronic inflammation accompanying obesity. High-density lipoprotein (HDL) and apolipoprotein A-I (apoA-I) produce anti-inflammatory effects via reverse cholesterol transport. However, the underlying mechanisms by which HDL and apoA-I inhibit inflammatory responses in adipocytes remain to be determined. Here we examined whether palmitate increases the translocation of TLR4 into lipid rafts and whether HDL and apoA-I inhibit inflammation in adipocytes. Palmitate exposure (250 μM, 24 h) increased *interleukin-6* and *tumor necrosis factor-α* gene expressions and translocation of TLR4 into lipid rafts in 3T3-L1 adipocytes. Pretreatment with HDL and apoA-I (50 μg/mL, 6 h) suppressed palmitate-induced inflammatory cytokine expression and TLR4 translocation into lipid rafts. Moreover, HDL and apoA-I inhibited palmitate-induced phosphorylation of nuclear factor-kappa B. HDL showed an anti-inflammatory effect via ATP-binding cassette transporter G1 and scavenger receptor class B, member 1, whereas apoA-I showed an effect via ATP-binding cassette transporter A1. These results demonstrated that HDL and apoA-I reduced palmitate-potentiated TLR4 trafficking into lipid rafts and its related inflammation in adipocytes via these specific transporters.

3.2673 Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic Escherichia coli O157: Intracellular delivery, trafficking and mechanisms of cell injury
Bielaszewska, M. et al
PloS Pathogens, ***13(2)****, e1006159 (2017)*

Outer membrane vesicles (OMVs) are important tools in bacterial virulence but their role in the pathogenesis of infections caused by enterohemorrhagic *Escherichia coli* (EHEC) O157, the leading cause of life-threatening hemolytic uremic syndrome, is poorly understood. Using proteomics, electron and confocal laser scanning microscopy, immunoblotting, and bioassays, we investigated OMVs secreted by EHEC O157 clinical isolates for virulence factors cargoes, interactions with pathogenetically relevant human cells, and mechanisms of cell injury. We demonstrate that O157 OMVs carry a cocktail of key virulence factors of EHEC O157 including Shiga toxin 2a (Stx2a), cytolethal distending toxin V (CdtV), EHEC hemolysin, and flagellin. The toxins are internalized by cells via dynamin-dependent endocytosis of OMVs and differentially separate from vesicles during intracellular trafficking. Stx2a and CdtV-B, the DNase-like CdtV subunit, separate from OMVs in early endosomes. Stx2a is trafficked, in association with its receptor globotriaosylceramide within detergent-resistant membranes, to the Golgi complex and the endoplasmic reticulum from where the catalytic Stx2a A1 fragment is translocated to the cytosol. CdtV-B is, after its retrograde transport to the endoplasmic reticulum, translocated to the nucleus to reach DNA. CdtV-A and CdtV-C subunits remain OMV-associated and are sorted with OMVs to lysosomes. EHEC hemolysin separates from OMVs in lysosomes and targets mitochondria. The OMV-delivered CdtV-B causes cellular DNA damage, which activates DNA damage responses leading to G2 cell cycle arrest. The arrested cells ultimately die of apoptosis induced by Stx2a and CdtV via caspase-9 activation. By demonstrating that naturally secreted EHEC O157 OMVs carry and deliver into cells a cocktail of biologically active virulence factors, thereby causing cell death, and by performing first comprehensive analysis of intracellular trafficking of OMVs and OMV-delivered virulence factors, we provide new insights into the pathogenesis of EHEC O157 infections. Our data have implications for considering O157 OMVs as vaccine candidates.

3.2674 Extracellular Vesicles: Unique Intercellular Delivery Vehicles
Maas, S.L.N., Breakfield, X.O. and Weaver, A.M.
*Trends in Cell Biol., **27(3)**, 172-188 (2017)*

Extracellular vesicles (EVs) are a heterogeneous collection of membrane-bound carriers with complex cargoes including proteins, lipids, and nucleic acids. While the release of EVs was previously thought to be only a mechanism to discard nonfunctional cellular components, increasing evidence implicates EVs as key players in intercellular and even interorganismal communication. EVs confer stability and can direct their cargoes to specific cell types. EV cargoes also appear to act in a combinatorial manner to communicate directives to other cells. This review focuses on recent findings and knowledge gaps in the area of EV biogenesis, release, and uptake. In addition, we highlight examples whereby EV cargoes control basic cellular functions, including motility and polarization, immune responses, and development, and contribute to diseases such as cancer and neurodegeneration.

3.2675 Glycan region of GPI anchored-protein is required for cytocidal oligomerization of an anticancer parasporin-2, Cry46Aa1 protein, from Bacillus thuringiensis strain A1547
Abe, Y., Inoue, H., Ashida, H., Maeda, Y., Kinoshita, T. and Kitada, S.
*J. Inverterbrate Pathol., **142**, 71-81 (2017)*

Parasporin-2 (PS2), alternatively named Cry46Aa1, an anticancer protein derived from Bacillus thuringiensis strain A1547, causes specific cell damage via PS2 oligomerization in the cell membrane. Although PS2 requires glycosylphosphatidylinositol (GPI)-anchored proteins for its cytocidal action, their precise role is unknown. Here, we report that the glycan of GPI induces PS2 oligomerization, which causes cell death. Cytotoxicity, cell-binding and oligomerization of the toxin were not observed in GPI-anchored protein-deficient Chinese hamster ovary cells. Expression and protease-treatment analyses showed that the actions of the toxin were dependent on the glycan core, not the polypeptide moiety, of GPI-anchored proteins. However, surface expression of some GPI-anchored proteins is observed in PS2-insensitive cells. These data suggest that GPI-anchored proteins do not determine the target specificity, but instead function as a kind of coreceptor, in the cytocidal action of PS2.

3.2676 Inhibition of Transient Receptor Potential Channel Mucolipin-1 (TRPML1) by Lysosomal Adenosine Involved in Severe Combined Immunodeficiency Diseases
Zhong, X.Z., Zou, Y., Sun, X., Dong, G., Cao, Q., Pandey, A., Rainey, J.K., Zhu, X. and Dong, X-P.
*J.Biol. Chem., **292(8)**, 3445-3455 (2017)*

Impaired adenosine homeostasis has been associated with numerous human diseases. Lysosomes are referred to as the cellular recycling centers that generate adenosine by breaking down nucleic acids or ATP. Recent studies have suggested that lysosomal adenosine overload causes lysosome defects that phenocopy patients with mutations in transient receptor potential channel mucolipin-1 (TRPML1), a lysosomal Ca^{2+} channel, suggesting that lysosomal adenosine overload may impair TRPML1 and then lead to subsequent lysosomal dysfunction. In this study, we demonstrate that lysosomal adenosine is elevated by deleting adenosine deaminase (ADA), an enzyme responsible for adenosine degradation. We also show that lysosomal adenosine accumulation inhibits TRPML1, which is rescued by overexpressing ENT3, the adenosine transporter situated in the lysosome membrane. Moreover, ADA deficiency results in lysosome enlargement, alkalinization, and dysfunction. These are rescued by activating TRPML1. Importantly, ADA-deficient B-lymphocytes are more vulnerable to oxidative stress, and this was rescued by TRPML1 activation. Our data suggest that lysosomal adenosine accumulation impairs lysosome function by inhibiting TRPML1 and subsequently leads to cell death in B-lymphocytes. Activating TRPML1 could be a new therapeutic strategy for those diseases.

3.2677 Sequences within the C Terminus of the Metabotropic Glutamate Receptor 5 (mGluR5) Are Responsible for Inner Nuclear Membrane Localization
Sergin, I., Jong, Y-J.I., Harmon, S.K., Kumar, V. and O'Malley, K.L.
*J. Biol. Chem., **292(9)**, 3637-3655 (2017)*

Traditionally, G-protein-coupled receptors (GPCR) are thought to be located on the cell surface where they transmit extracellular signals to the cytoplasm. However, recent studies indicate that some GPCRs are also localized to various subcellular compartments such as the nucleus where they appear required for various biological functions. For example, the metabotropic glutamate receptor 5 (mGluR5) is concentrated at the inner nuclear membrane (INM) where it mediates Ca^{2+} changes in the nucleoplasm by coupling with $G_{q/11}$.

Here, we identified a region within the C-terminal domain (amino acids 852–876) that is necessary and sufficient for INM localization of the receptor. Because these sequences do not correspond to known nuclear localization signal motifs, they represent a new motif for INM trafficking. mGluR5 is also trafficked to the plasma membrane where it undergoes re-cycling/degradation in a separate receptor pool, one that does not interact with the nuclear mGluR5 pool. Finally, our data suggest that once at the INM, mGluR5 is stably retained via interactions with chromatin. Thus, mGluR5 is perfectly positioned to regulate nucleoplasmic Ca^{2+} *in situ*.

3.2678 Methods to isolate extracellular vesicles for diagnosis
Kang, H., Kim, J. and Park, J.
*Micro and Nano Syst. Lett., **5**:15 82017)*

Extracellular vesicles (EVs) are small membrane-bound bodies that are released into extracellular space by diverse cells, and are found in body fluids like blood, urine and saliva. EVs contain RNA, DNA and proteins, which can be biomarkers for diagnosis. EVs can be obtained by minimally-invasive biopsy, so they are useful in disease diagnosis. High yield and purity contribute to precise diagnosis of disease, but damaged EVs and impurities can cause confu sed results. However, EV isolation methods have different yields and purities. Furthermore, the isolation method that is most suitable to maximize EV recovery efficiency depends on the experimental conditions. This review focuses on merits and demerits of several types of EV isolation methods, and provides examples of how to diagnose disease by exploiting information obtained by analysis of EVs.

3.2679 Misrouting of v-ATPase subunit V0a1 dysregulates lysosomal acidification in a neurodegenerative lysosomal storage disease model
Bagh, M.B., Peng, S., Chandra, G., Zhang, Z., Singh, S.P., pattabiraman, N., Liu, A. and Mukherjee, A.B.
*Nature Communications, **8**:14612 (2017)*

Defective lysosomal acidification contributes to virtually all lysosomal storage disorders (LSDs) and to common neurodegenerative diseases like Alzheimer's and Parkinson's. Despite its fundamental importance, the mechanism(s) underlying this defect remains unclear. The v-ATPase, a multisubunit protein complex composed of cytosolic V1-sector and lysosomal membrane-anchored V0-sector, regulates lysosomal acidification. Mutations in the CLN1 gene, encoding PPT1, cause a devastating neurodegenerative LSD, INCL. Here we report that in Cln1−/− mice, which mimic INCL, reduced v-ATPase activity correlates with elevated lysosomal pH. Moreover, v-ATPase subunit a1 of the V0 sector (V0a1) requires palmitoylation for interacting with adaptor protein-2 (AP-2) and AP-3, respectively, for trafficking to the lysosomal membrane. Notably, treatment of Cln1−/− mice with a thioesterase (Ppt1)-mimetic, NtBuHA, ameliorated this defect. Our findings reveal an unanticipated role of Cln1 in regulating lysosomal targeting of V0a1 and suggest that varying factors adversely affecting v-ATPase function dysregulate lysosomal acidification in other LSDs and common neurodegenerative diseases.

3.2680 Sorting Nexin 9 facilitates podocin endocytosis in the injured podocyte
Sasaki, Y., Hidaka, T., Ueno, T., Akiba-Takagi, M., Trejo, J.A.O., Seki, T., Nagai-Hosoe, Y., Tanaka, E., Horikoshi, S., Tomino, Y., Suzuki, Y. and Asanuma, K.
*Scientific Reports, **7**:43921 (2017)*

The irreversibility of glomerulosclerotic changes depends on the degree of podocyte injury. We have previously demonstrated the endocytic translocation of podocin to the subcellular area in severely injured podocytes and found that this process is the primary disease trigger. Here we identified the protein sorting nexin 9 (SNX9) as a novel facilitator of podocin endocytosis in a yeast two-hybrid analysis. SNX9 is involved in clathrin-mediated endocytosis, actin rearrangement and vesicle transport regulation. Our results revealed and confirmed that SNX9 interacts with podocin exclusively through the Bin–Amphiphysin–Rvs (BAR) domain of SNX9. Immunofluorescence staining revealed the expression of SNX9 in response to podocyte adriamycin-induced injury both in vitro and in vivo. Finally, an analysis of human glomerular disease biopsy samples demonstrated strong SNX9 expression and co-localization with podocin in samples representative of severe podocyte injury, such as IgA nephropathy with poor prognosis, membranous nephropathy and focal segmental glomerulosclerosis. In conclusion, we identified SNX9 as a facilitator of podocin endocytosis in severe podocyte injury and demonstrated the expression of SNX9 in the podocytes of both nephropathy model mice and human patients with irreversible glomerular disease.

3.2681 Defining the purity of exosomes required for diagnostic profiling of small RNA suitable for biomarker discovery

Quek, C., Bellingham, S.A., Jung, C-H., Scicluna, B.J., Shambrook, M.C., Sharples, R.A., Cheng, L. and Hill, A.F.

RNA Biol., ***14(2)****, 245-258 (2017)*

Small non-coding RNAs (ncRNA), including microRNAs (miRNA), enclosed in exosomes are being utilised for biomarker discovery in disease. Two common exosome isolation methods involve differential ultracentrifugation or differential ultracentrifugation coupled with Optiprep gradient fractionation. Generally, the incorporation of an Optiprep gradient provides better separation and increased purity of exosomes. The question of whether increased purity of exosomes is required for small ncRNA profiling, particularly in diagnostic and biomarker purposes, has not been addressed and highly debated. Utilizing an established neuronal cell system, we used next-generation sequencing to comprehensively profile ncRNA in cells and exosomes isolated by these 2 isolation methods. By comparing ncRNA content in exosomes from these two methods, we found that exosomes from both isolation methods were enriched with miRNAs and contained a diverse range of rRNA, small nuclear RNA, small nucleolar RNA and piwi-interacting RNA as compared with their cellular counterparts. Additionally, tRNA fragments (30-55 nucleotides in length) were identified in exosomes and may act as potential modulators for repressing protein translation. Overall, the outcome of this study confirms that ultracentrifugation-based method as a feasible approach to identify ncRNA biomarkers in exosomes.

3.2682 Plasmalogen biosynthesis is spatiotemporally regulated by sensing plasmalogens in the inner leaflet of plasma membranes

Honsho, M., Abe, Y. and Fujiki, Y.

Scientific Reports, ***7****:43936 (2017)*

Alkenyl ether phospholipids are a major sub-class of ethanolamine- and choline-phospholipids in which a long chain fatty alcohol is attached at the sn-1 position through a vinyl ether bond. Biosynthesis of ethanolamine-containing alkenyl ether phospholipids, plasmalogens, is regulated by modulating the stability of fatty acyl-CoA reductase 1 (Far1) in a manner dependent on the level of cellular plasmalogens. However, precise molecular mechanisms underlying the regulation of plasmalogen synthesis remain poorly understood. Here we show that degradation of Far1 is accelerated by inhibiting dynamin-, Src kinase-, or flotillin-1-mediated endocytosis without increasing the cellular level of plasmalogens. By contrast, Far1 is stabilized by sequestering cholesterol with nystatin. Moreover, abrogation of the asymmetric distribution of plasmalogens in the plasma membrane by reducing the expression of CDC50A encoding a β-subunit of flippase elevates the expression level of Far1 and plasmalogen synthesis without reducing the total cellular level of plasmalogens. Together, these results support a model that plasmalogens localised in the inner leaflet of the plasma membranes are sensed for plasmalogen homeostasis in cells, thereby suggesting that plasmalogen synthesis is spatiotemporally regulated by monitoring cellular level of plasmalogens.

3.2683 Critical role of quorum sensing-dependent glutamate metabolism in homeostatic osmolality and outer membrane vesiculation in Burkholderia glumae

Kang, Y., Goo, E., Kim, J. and Hwang, I.

Scientific Reports, ***7****:44195 (2017)*

Metabolic homeostasis in cooperative bacteria is achieved by modulating primary metabolism in a quorum sensing (QS)-dependent manner. A perturbed metabolism in QS mutants causes physiological stress in the rice bacterial pathogen Burkholderia glumae. Here, we show that increased bacterial osmolality in B. glumae is caused by unusually high cellular concentrations of glutamate and betaine generated by QS deficiencies. QS negatively controls glutamate uptake and the expression of genes involved in the glutamine synthetase and glutamine oxoglutarate aminotransferase cycles. Thus, cellular glutamate levels were significantly higher in the QS mutants than in the wild type, and they caused hyperosmotic cellular conditions. Under the hypotonic conditions of the periplasm in the QS mutants, outer membrane bulging and vesiculation were observed, although these changes were rescued by knocking out the gltI gene, which encodes a glutamate transporter. Outer membrane modifications were not detected in the wild type. These results suggest that QS-dependent glutamate metabolism is critical for homeostatic osmolality. We suggest that outer membrane bulging and vesiculation might be the outcome of a physiological adaptation to relieve hypotonic osmotic stress in QS mutants. Our findings reveal how QS functions to maintain bacterial osmolality in a cooperative population.

3.2684 Localization of Protein Kinase NDR2 to Peroxisomes and Its Role in Ciliogenesis
Abe, S., Nagai, T., Masukawa, M., Okumoto, K., Homma, Y., Fujiki, Y. and Mizuno, K.
J. Biol. Chem., ***292(10)***, *4089-4098 (2017)*

Nuclear Dbf2-related (NDR) kinases, comprising NDR1 and NDR2, are serine/threonine kinases that play crucial roles in the control of cell proliferation, apoptosis, and morphogenesis. We recently showed that NDR2, but not NDR1, is involved in primary cilium formation; however, the mechanism underlying their functional difference in ciliogenesis is unknown. To address this issue, we examined their subcellular localization. Despite their close sequence similarity, NDR2 exhibited punctate localization in the cytoplasm, whereas NDR1 was diffusely distributed within the cell. Notably, NDR2 puncta mostly co-localized with the peroxisome marker proteins, catalase and CFP-SKL (cyan fluorescent protein carrying the C-terminal typical peroxisome-targeting signal type-1 (PTS1) sequence, Ser-Lys-Leu). NDR2 contains the PTS1-like sequence, Gly-Lys-Leu, at the C-terminal end, whereas the C-terminal end of NDR1 is Ala-Lys. An NDR2 mutant lacking the C-terminal Leu, NDR2(ΔL), exhibited almost diffuse distribution in cells. Additionally, NDR2, but neither NDR1 nor NDR2(ΔL), bound to the PTS1 receptor Pex5p. Together, these findings indicate that NDR2 localizes to the peroxisome by using the C-terminal GKL sequence. Intriguingly, topology analysis of NDR2 suggests that NDR2 is exposed to the cytosolic surface of the peroxisome. The expression of wild-type NDR2, but not NDR2(ΔL), recovered the suppressive effect of NDR2 knockdown on ciliogenesis. Furthermore, knockdown of peroxisome biogenesis factor genes (*PEX1* or *PEX3*) partially suppressed ciliogenesis. These results suggest that the peroxisomal localization of NDR2 is implicated in its function to promote primary cilium formation.

3.2685 Comparative Characterization of Phosphatidic Acid Sensors and Their Localization during Frustrated Phagocytosis
Kassas, N., Tanguy, E., Thahouly, T., Fouillen, L., Heintz, D., Chasserot-Golaz, S., Bader, M-F., grant, N.J. and Vitale, N.
J. Biol. Chem., ***292(10)***, *4266-4279 (2017)*

Phosphatidic acid (PA) is the simplest phospholipid naturally existing in living organisms, but it constitutes only a minor fraction of total cell lipids. PA has attracted considerable attention because it is a phospholipid precursor, a lipid second messenger, and a modulator of membrane shape, and it has thus been proposed to play key cellular functions. The dynamics of PA in cells and in subcellular compartments, however, remains an open question. The recent generation of fluorescent probes for PA, by fusing GFP to PA-binding domains, has provided direct evidence for PA dynamics in different intracellular compartments. Here, three PA sensors were characterized *in vitro*, and their preferences for different PA species in particular lipidic environments were compared. In addition, the localization of PA in macrophages during frustrated phagocytosis was examined using these PA sensors and was combined with a lipidomic analysis of PA in intracellular compartments. The results indicate that the PA sensors display some preferences for specific PA species, depending on the lipid environment, and the localization study in macrophages revealed the complexity of intracellular PA dynamics.

3.2686 Multiplex gene editing by CRISPR–Cpf1 using a single crRNA array
Zetsche, B. et al
Nature Biotechnology, ***35(1)***, *31-34 (2017)*

Targeting of multiple genomic loci with Cas9 is limited by the need for multiple or large expression constructs. Here we show that the ability of Cpf1 to process its own CRISPR RNA (crRNA) can be used to simplify multiplexed genome editing. Using a single customized CRISPR array, we edit up to four genes in mammalian cells and three in the mouse brain, simultaneously.

3.2687 Tyrosine phosphorylation modulates mitochondrial chaperonin Hsp60 and delays rotavirus NSP4-mediated apoptotic signaling in host cells
Chattopadhyay, S., Mukherjee, A., Patra, U., Bhowmick, R., Basak, T., Sengupta, S. and Chawla-Sarkar, M.
Cell. Microbiol., ***19(3)***, *e12670 (2017)*

Phosphoproteomics-based platforms have been widely used to identify post translational dynamics of cellular proteins in response to viral infection. The present study was undertaken to assess differential tyrosine phosphorylation during early hours of rotavirus (RV) SA11 infection. Heat shock proteins

(Hsp60) were found to be enriched in the data set of RV-SA11 induced differentially tyrosine-phosphorylated proteins at 2 hr post infection (hpi). Hsp60 was further found to be phosphorylated by an activated form of Src kinase on 227th tyrosine residue, and tyrosine phosphorylation of mitochondrial chaperonin Hsp60 correlated with its proteasomal degradation at 2–2.5hpi. Interestingly, mitochondrial Hsp60 positively influenced translocation of the rotaviral nonstructural protein 4 to mitochondria during RV infections. Phosphorylation and subsequent transient degradation of mitochondrial Hsp60 during early hours of RV-SA11 infection resulted in inhibition of premature import of nonstructural protein 4 into mitochondria, thereby delaying early apoptosis. Overall, the study highlighted one of the many strategies rotavirus undertakes to prevent early apoptosis and subsequent reduced viral progeny yield.

3.2688 Extracellular vesicles: their role in cancer biology and epithelial-mesenchymal transition
Gopal, S.K., Greening, D.W., Rai, A., Chen, M., Xu, R., Shafiq, A., Mathias, R.A., Zhu, H-J., and Simpson, R.J.
Biochemical J., ***474(1)***, *21-45 (2017)*

Cell–cell communication is critical across an assortment of physiological and pathological processes. Extracellular vesicles (EVs) represent an integral facet of intercellular communication largely through the transfer of functional cargo such as proteins, messenger RNAs (mRNAs), microRNA (miRNAs), DNAs and lipids. EVs, especially exosomes and shed microvesicles, represent an important delivery medium in the tumour micro-environment through the reciprocal dissemination of signals between cancer and resident stromal cells to facilitate tumorigenesis and metastasis. An important step of the metastatic cascade is the reprogramming of cancer cells from an epithelial to mesenchymal phenotype (epithelial–mesenchymal transition, EMT), which is associated with increased aggressiveness, invasiveness and metastatic potential. There is now increasing evidence demonstrating that EVs released by cells undergoing EMT are reprogrammed (protein and RNA content) during this process. This review summarises current knowledge of EV-mediated functional transfer of proteins and RNA species (mRNA, miRNA, long non-coding RNA) between cells in cancer biology and the EMT process. An in-depth understanding of EVs associated with EMT, with emphasis on molecular composition (proteins and RNA species), will provide fundamental insights into cancer biology.

3.2689 RasGRF Couples Nox4-Dependent Endoplasmic Reticulum Signaling to Ras
Wu, R.F., Liao, C., Hatoum, H., Fu, G., Ochoa, C.D. and Terada, L.S.
Arterioscler. Thromb. Vasc. Biol., ***37(1)***, *98-107 (2017)*

Objectives—In response to endoplasmic reticulum (ER) stress, endothelial cells initiate corrective pathways such as the unfolded protein response. Recent studies suggest that reactive oxygen species produced on the ER may participate in homeostatic signaling through Ras in response to ER stress. We sought to identify mechanisms responsible for this focal signaling pathway.
Approach and Results—In endothelial cells, we found that ER stress induced by tunicamycin activates the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase Nox4 focally on the ER surface but not on the plasma membrane. Ras activation is also restricted to the ER, occurs downstream of Nox4, and is required for activation of the unfolded protein response. In contrast, treatment with the growth factor VEGF (vascular endothelial growth factor) results in Ras activation and reactive oxygen species production confined instead to the plasma membrane and not to the ER, demonstrating local coupling of reactive oxygen species and Ras signals. We further identify the calcium-responsive, ER-resident guanyl exchange factors RasGRF1 and RasGRF2 as novel upstream mediators linking Nox4 with Ras activation in response to ER stress. Oxidation of the sarcoendoplasmic reticulum calcium ATPase and increases in cytosolic calcium caused by ER stress are blocked by Nox4 knockdown, and reduction in cytosolic free calcium prevents both Ras activation and the unfolded protein response.
Conclusions—ER stress triggers a localized signaling module on the ER surface involving Nox4-dependent calcium mobilization, which directs local Ras activation through ER-associated, calcium-responsive RasGRF.

3.2690 Chapter Two – Rationally Designed Peptide Probes for Extracellular Vesicles
Tamura, R. and Yin, H.
Adv. Clin. Chem., ***79***, *25-41 (2017)*

Extracellular vesicles (EVs) are submicroscopic lipid vesicles secreted from cells and play significant roles in cell-to-cell communication by transporting varieties of cell signaling molecules like proteins, DNA, mRNA, and microRNA. Recent studies showed that EVs are highly correlated with cancer progression and

metastasis. However, there are some difficulties in probing each vesicle using popular analytical methods because of their small sizes and heterogeneous origins. These obstacles may be overcome by using a novel approach that senses highly curved membrane and negatively charged membrane lipids. In this chapter, we highlight the basic biological concepts of EVs, isolation, and quantification methods, and recent advent of peptide probes for EVs.

3.2691 Membrane vesicles in sea water: heterogeneous DNA content and implications for viral abundance estimates

Biller, S.J., McDaniel, L.D., Breitbart, M., Rogers, E., Paul, J.H. and Chrisholm, S.W.
ISME J., ***11***, *394-404 (2017)*

Diverse microbes release membrane-bound extracellular vesicles from their outer surfaces into the surrounding environment. Vesicles are found in numerous habitats including the oceans, where they likely have a variety of functional roles in microbial ecosystems. Extracellular vesicles are known to contain a range of biomolecules including DNA, but the frequency with which DNA is packaged in vesicles is unknown. Here, we examine the quantity and distribution of DNA associated with vesicles released from five different bacteria. The average quantity of double-stranded DNA and size distribution of DNA fragments released within vesicles varies among different taxa. Although some vesicles contain sufficient DNA to be visible following staining with the SYBR fluorescent DNA dyes typically used to enumerate viruses, this represents only a small proportion (<0.01–1%) of vesicles. Thus DNA is packaged heterogeneously within vesicle populations, and it appears that vesicles are likely to be a minor component of SYBR-visible particles in natural sea water compared with viruses. Consistent with this hypothesis, chloroform treatment of coastal and offshore seawater samples reveals that vesicles increase epifluorescence-based particle (viral) counts by less than an order of magnitude and their impact is variable in space and time.

3.2692 Large-scale isolation and cytotoxicity of extracellular vesicles derived from activated human natural killer cells

Jong, A.Y., Wu, C-H., Li, J., Sun, J., Fabbri, M., Wayne, A.S. and Seeger, R.C.
J. Extracellular Vesicles, ***6****:1, 1294368 (2017)*

Extracellular vesicles (EVs) have been the focus of great interest, as they appear to be involved in numerous important cellular processes. They deliver bioactive macromolecules such as proteins, lipids, and nucleic acids, allowing intercellular communication in multicellular organisms. EVs are secreted by all cell types, including immune cells such as natural killer cells (NK), and they may play important roles in the immune system. Currently, a large-scale procedure to obtain functional NK EVs is lacking, limiting their use clinically. In this report, we present a simple, robust, and cost-effective method to isolate a large quantity of NK EVs. After propagating and activating NK cells *ex vivo* and then incubating them in exosome-free medium for 48 h, EVs were isolated using a polymer precipitation method. The isolated vesicles contain the tetraspanin CD63, an EV marker, and associated proteins (fibronectin), but are devoid of cytochrome C, a cytoplasmic marker. Nanoparticle tracking analysis showed a size distribution between 100 and 200 nm while transmission electron microscopy imaging displayed vesicles with an oval shape and comparable sizes, fulfilling the definition of EV. Importantly, isolated EV fractions were cytotoxic against cancer cells. Furthermore, our results demonstrate for the first time that isolated activated NK (aNK) cell EVs contain the cytotoxic proteins perforin, granulysin, and granzymes A and B, incorporated from the aNK cells. Activation of caspase -3, -7 and -9 was detected in cancer cells incubated with aNK EVs, and caspase inhibitors blocked aNK EV-induced cytotoxicity, suggesting that aNK EVs activate caspase pathways in target cells. The ability to isolate functional aNK EVs on a large scale may lead to new clinical applications.

3.2693 Testosterone promotes tube formation of endothelial cells isolated from veins via activation of Smad1 protein

Liu, P., Li, X., Song, F., Li, P., Wei, J., Yan, Q., Xu, X., Yang, J., Li, C. and Fu, X.
Mol. Cell. Endocrinol., ***446***, *21-31 (2017)*

Testosterone (T) deficiency is positively correlated with the increased incidence of cardiovascular disease. However, the effects of T on vascular endothelial cells remain obscure. Tube formation capacity is critical for vascular regeneration/repair and Smad1 plays an important role in these events. In this study, we investigated the effects of T on Smad1 activation and tube formation of cultured human umbilical endothelial cells (HUVECs). Our results showed that T rapidly increased endothelial Smad1

phosphorylation. This effect was mimicked by cell-impermeable T-BSA conjugates and was not altered by transcriptional inhibitor actinomycin D or translational inhibitor cycloheximide. T-induced Smad1 phosphorylation was blocked by ERK1/2 and c-Src inhibitors or their specific siRNAs, while it was reinforced by ERK1/2 or c-Src overexpression. Indeed, T rapidly activated ERK1/2 and c-Src signalings and c-Src was confirmed as the upstream of ERK1/2. Moreover, caveolae disruptor methyl-β-cyclodextrin (β-MCD) blocked Smad1 activation induced by T. The association of caveolin-1 with androgen receptor (AR) or c-Src was detected by immunoprecipitation and it was significantly increased by rapid T stimulation. Furthermore, fractional analysis showed that AR and c-Src were expressed in caveolae-enriched membrane fractions. T promoted tube formation of HUVECs, which was blocked by c-Src and ERK1/2 inhibitors or by the knockdown of Smad1. In conclusion, T increased tube formation of endothelial cells isolated from veins by stimulating Smad1 phosphorylation in a nongenomic manner, which was mediated by signals from AR/c-Src located in caveolae to ERK1/2 cascade. These findings may shed new light on the relevance of T to its vascular functions.

3.2694 LOXL2 drives epithelial-mesenchymal transition via activation of IRE1-XBP1 signalling pathway
Cuevas, E.P., Eraso, P., Mazon, M.J:, Santos, V., Moreno-Bueno, G., Cano, A. and Portillo, F.
*Scientific Reports, **7**:44988 (2017)*

Epithelial-to-Mesenchymal Transition (EMT) is a key process contributing to the aggressiveness of cancer cells. EMT is triggered by activation of different transcription factors collectively known as EMT-TFs. Different cellular cues and cell signalling networks activate EMT at transcriptional and posttranscriptional level in different biological and pathological situations. Among them, overexpression of LOXL2 (lysyl oxidase-like 2) induces EMT independent of its catalytic activity. Remarkably, perinuclear/cytoplasmic accumulation of LOXL2 is a poor prognosis marker of squamous cell carcinomas and is associated to basal breast cancer metastasis by mechanisms no yet fully understood. Here, we report that overexpression of LOXL2 promotes its accumulation in the Endoplasmic Reticulum where it interacts with HSPA5 leading to activation of the IRE1-XBP1 signalling pathway of the ER-stress response. LOXL2-dependent IRE1-XBP1 activation induces the expression of several EMT-TFs: SNAI1, SNAI2, ZEB2 and TCF3 that are direct transcriptional targets of XBP1. Remarkably, inhibition of IRE1 blocks LOXL2-dependent upregulation of EMT-TFs thus hindering EMT induction.

3.2695 623 – Purification of urinary extracellular vesicles for uro-oncological biomarker studies using an iodixanol (Optiprep™) density gradient
Dhondt, B., Vergauwen, G., Van Deun, J., Geeurickx, E., Claeys, T., Poelaert, F., Buelens, s., Hendrix, A., De Wever, O. and Lumen, N.
*Eur. Urol. Suppl., **16(3)**, e1078 (2017)*

INTRODUCTION & OBJECTIVES: Extracellular vesicles (EVs), are bilayered, nanometer-sized vesicles
released by cells into the extracellular space after fusion of multivesicular endosomes with the plasma membrane or by direct budding. They play a role in intercellular communication by transporting proteins and nucleic acids to target cells. Their molecular content is a fingerprint of the releasing cells. In addition, EVs are released into easily accessible body fluids such as urine. EV analyses can therefore be considered as a real-time "liquid biopsy" for patients with cancer. Consequently, urinary EVs (uEVs) might be used to diagnose urological cancers or to monitor progression and therapy response. The isolation of highly purified uEVs is a prerequisite to obtain reliable omics data for biomarker studies. Tamm-Horsfall protein (THP; uromodulin) polymers in urine are co-isolated as a contaminant when the current EV isolation methods are applied, hindering accurate downstream analysis by masking of low abundance proteins or by entrapment of non-EV associated extracellular RNA molecules. For biomarker studies, the MISEV (Minimal Information for Studies on Extracellular Vesicles) criteria encourage the use of density gradients to at least validate the association of the identified molecules with EVs. This study evaluates the ability of an iodixanol (OptiprepTM) density gradient to separate uEVs from THP contamination.
MATERIAL & METHODS: Urine samples of prostate cancer patients were collected, pooled and centrifuged for 10 minutes at 1000g before storage at -80° C. Upon use, pooled samples of 50 ml were thawed at room temperature and concentrated using a Centricon® Plus-70 Centrifugal Filter Device with a 10kD MWCO. Using an OptiprepTM density gradient (ODG), uEVs were isolated, applying both a topdown
(sedimentation) and a bottom-up (floatation) approach. Yield and size distribution of the isolated particles were assessed by nanoparticle tracking analysis (NTA). The successful isolation of uEVs was

evaluated by western blot (WB) analysis, assessing the presence of the EV-associated protein markers ALIX, TSG101 and CD9. THP contamination was assessed by Ponceau S staining and confirmed by WB.
RESULTS: In top-down ODG, Ponceau S staining identified THP as a smear over the different ODG fractions. This was verified and supported by WB. Surprisingly, the highest amount of THP was seen in the ODG fractions containing the uEVs. In a bottom-up ODG approach, the abundance of THP was found in the bottom fractions of the gradient. Ponceau S staining did not identify the presence of THP in the EV rich fraction of the bottom-up ODG. Conversely, WB analysis did identify residual presence of THP in the EV rich fraction. In addition, WB demonstrated the presence of EV-associated proteins in the bottom fractions, indicating uEV entrapment by THP complexes.
CONCLUSIONS: Bottom-up ODG results in an efficient, but not complete, separation of uEVs from THP
contamination. Further research should evaluate if adding additional purification steps results in obtaining highly purified EVs from urine in order to conduct reliable uro-oncological biomarker studies.

3.2696 Oxygen tension regulates the miRNA profile and bioactivity of exosomes released from extravillous trophoblast cells – Liquid biopsies for monitoring complications of pregnancy
Truong, G., Guanzon, D., Kinhal, V., Elfeky, O., Lai, A., Longo, S., Nuzhat, Z., Palma, C., Scholz-Romero, K., Menon, R., Mol, B.W., Rice, G.E. and Salomon, C.
*PloS One, **12(3)**, e0174514 (2017)*

Our understanding of how cells communicate has undergone a paradigm shift since the recent recognition of the role of exosomes in intercellular signaling. In this study, we investigated whether oxygen tension alters the exosome release and miRNA profile from extravillous trophoblast (EVT) cells, modifying their bioactivity on endothelial cells (EC). Furthermore, we have established the exosomal miRNA profile at early gestation in women who develop pre-eclampsia (PE) and spontaneous preterm birth (SPTB). HTR-8/SVneo cells were used as an EVT model. The effect of oxygen tension (i.e. 8% and 1% oxygen) on exosome release was quantified using nanocrystals (Qdot®) coupled to CD63 by fluorescence NTA. A real-time, live-cell imaging system (Incucyte™) was used to establish the effect of exosomes on EC. Plasma samples were obtained at early gestation (<18 weeks) and classified according to pregnancy outcomes. An Illumina TrueSeq Small RNA kit was used to construct a small RNA library from exosomal RNA obtained from EVT and plasma samples. The number of exosomes was significantly higher in EVT cultured under 1% compared to 8% oxygen. In total, 741 miRNA were identified in exosomes from EVT. Bioinformatic analysis revealed that these miRNA were associated with cell migration and cytokine production. Interestingly, exosomes isolated from EVT cultured at 8% oxygen increased EC migration, whilst exosomes cultured at 1% oxygen decreased EC migration. These changes were inversely proportional to TNF-α released from EC. Finally, we have identified a set of unique miRNAs in exosomes from EVT cultured at 1% oxygen and exosomes isolated from the circulation of mothers at early gestation, who later developed PE and SPTB. We suggest that aberrant exosomal signalling by placental cells is a common aetiological factor in pregnancy complications characterised by incomplete SpA remodeling and is therefore a clinically relevant biomarker of pregnancy complications.

3.2697 Sorting nexin-4 regulates β-amyloid production by modulating β-site-activating cleavage enzyme-1
Kim, N-Y., Cho, M-H., Won, S-H., Kang, H-J., Yoon, S-Y. and Kim, D-H.
Alzheimer Res. & Therapy, 9:4 (2017)

Background
Amyloid precursor protein (APP) is cleaved by β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) to produce β-amyloid (Aβ), a critical pathogenic peptide in Alzheimer's disease (AD). Aβ generation can be affected by the intracellular trafficking of APP or its related secretases, which is thus important to understanding its pathological alterations. Although sorting nexin (SNX) family proteins regulate this trafficking, the relevance and role of sorting nexin-4 (SNX4) regarding AD has not been studied yet.
Methods
In this study, human brain tissue and APP/PS1 mouse brain tissue were used to check the disease relevance of SNX4. To investigate the role of SNX4 in AD pathogenesis, several experiments were done, such as coimmunoprecipitation, Western blotting, immunohistochemistry, and gradient fractionation.
Results
We found that SNX4 protein levels changed in the brains of patients with AD and of AD model mice. Overexpression of SNX4 significantly increased the levels of BACE1 and Aβ. Downregulation of SNX4

had the opposite effect. SNX4 interacts with BACE1 and prevents BACE1 trafficking to the lysosomal degradation system, resulting in an increased half-life of BACE1 and increased production of Aβ.
Conclusions
We show that SNX4 regulates BACE1 trafficking. Our findings suggest novel therapeutic implications of modulating SNX4 to regulate BACE1-mediated β-processing of APP and subsequent Aβ generation.

3.2698 Mitochondria are devoid of amyloid β-protein (Aβ)-producing secretases: Evidence for unlikely occurrence within mitochondria of Aβ generation from amyloid precursor protein
Mamada, N., Tanokashira, D., Ishii, K., Tamaoka, A. and Araki, W.
Biochem. Biophys. Res.Comm., ***486****, 321-328 (2017)*

Mitochondrial dysfunction is implicated in the pathological mechanism of Alzheimer's disease (AD). Amyloid β-protein (Aβ), which plays a central role in AD pathogenesis, is reported to accumulate within mitochondria. However, a question remains as to whether Aβ is generated locally from amyloid precursor protein (APP) within mitochondria. We investigated this issue by analyzing the expression patterns of APP, APP-processing secretases, and APP metabolites in mitochondria separated from human neuroblastoma SH-SY5Y cells and those expressing Swedish mutant APP. APP, BACE1, and PEN-2 protein levels were significantly lower in crude mitochondria than microsome fractions while those of ADAM10 and the other γ-secretase complex components (presenilin 1, nicastrin, and APH-1) were comparable between fractions. The crude mitochondrial fraction containing substantial levels of cathepsin D, a lysosomal marker, was further separated via iodixanol gradient centrifugation to obtain mitochondria- and lysosome-enriched fractions. Mature APP, BACE1, and all γ-secretase complex components (in particular, presenilin 1 and PEN-2) were scarcely present in the mitochondria-enriched fraction, compared to the lysosome-enriched fraction. Moreover, expression of the β-C-terminal fragment (β-CTF) of APP was markedly low in the mitochondria-enriched fraction. Additionally, immunocytochemical analysis showed very little co-localization between presenilin 1 and Tom20, a marker protein of mitochondria. In view of the particularly low expression levels of BACE1, γ-secretase complex proteins, and β-CTF in mitochondria, we propose that it is unlikely that Aβ generation from APP occurs locally within this organelle.

3.2699 Glycoconjugates from extracellular vesicles: Structures, functions and emerging potential as cancer biomarkers
Costa, J.
*BBA – Reviews on Cancer****, 1868****, 157-166 (2017)*

Extracellular vesicles (EVs) are released by virtually all cells, carry cellular molecules to the extracellular environment, and may interact with other cells. They are found in body fluids, therefore, constituting useful target sources for the identification of disease biomarkers, for example, in cancer. EVs originate from the plasma membrane or from multivesicular endosomes. They have the same topology as the plasma membrane and are rich in glycoconjugates, displaying specific glycosignatures. Surface glycoconjugates play important roles in EVs biogenesis and in their interaction with other cells. Changes in glycosylation constitute a hallmark of different types of cancer, therefore, the study of glycoconjugates and glycosignatures of EVs appear as promising candidates to identify novel cancer biomarkers and to increase the specificity and sensitivity of the existing clinical biomarkers, many of which are glycosylated.

3.2700 Assessment of Caveolae/Lipid Rafts in Isolated Cells
Callera, G.E., Bruder-Nascimento, T. and Touyz, R.M.
Methods in Mol. Biol., ***1527****, 251-269 (2017)*

This chapter outlines protocols to evaluate protein localization, recruitment or phosphorylation levels in cholesterol/sphingolipids-enriched cell membrane domains and recommends experimental designs with pharmacological tolls to evaluate potential cell functions associated with these domains. We emphasize the need for the combination of several approaches towards understanding the protein components and cellular functions attributed to these distinct microdomains.

3.2701 A Protocol for Isolation and Proteomic Characterization of Distinct Extracellular Vesicle Subtypes by Sequential Centrifugal Ultrafiltration
Xu, R., Simpson, R.J. and Greening, D.W.
Methods in Mol. Biol., ***1545****, 91-116 (2017)*

Scientific and clinical interest in extracellular vesicles (EVs) has increased rapidly as evidence mounts that they may constitute a new signaling paradigm. Recent studies have highlighted EVs carry preassembled complex biological information that elicit pleiotropic responses in target cells. It is well recognized that cells secrete essentially two EV subtypes that can be partially separated by differential centrifugation (DC): the larger size class (referred to as "microvesicles" or "shed microvesicles," sMVs) is heterogeneous (100–1500 nm), while the smaller size class (referred to as "exosomes") is relatively homogeneous in size (50–150 nm). A key issue hindering progress in understanding underlying mechanisms of EV subtype biogenesis and cargo selectivity has been the technical challenge of isolating homogeneous EV subpopulations suitable for molecular analysis. In this protocol we reveal a novel method for the isolation, purification, and characterization of distinct EV subtypes: exosomes and sMVs. This method, based on sequential centrifugal ultrafiltration (SCUF), affords unbiased isolation of EVs from conditioned medium from a human colon cancer cell model. For both EV subtypes, this protocol details extensive purification and characterization based on dynamic light scattering, cryoelectron microscopy, quantitation, immunoblotting, and comparative label-free proteome profiling. This analytical SCUF method developed is potentially scalable using tangential flow filtration and provides a solid foundation for future in-depth functional studies of EV subtypes from diverse cell types.

3.2702 Isolation of Exosomes from HTLV-Infected Cells
Barclay, R.A., Pleet, M.L., Akpamagbo, Y., Noor, K., Mathiesen, A. and Kashanchi, F.
Methods in Mol. Biol., ***1582***, *57-75 (2017)*

Exosomes are small vesicles, approximately 30–100 nm in diameter, that transport various cargos, such as proteins and nucleic acids, between cells. It has been previously shown that exosomes can also transport viral proteins, such as the HTLV protein Tax, and viral RNAs, potentially contributing to disease pathogenesis. Therefore, it is important to understand their impact on recipient cells. Here, we describe methods of isolating and purifying exosomes from cell culture or tissue through ultracentrifugation, characterizing exosomes by surface biomarkers, and assays that evaluate the effect of exosomes on cells.

3.2703 Quantitative Analysis of Exosomal miRNA via qPCR and Digital PCR
Bellingham, S.A., Shambrook, M. and Hill, A.F.
Methods in Mol. Biol., ***1545***, *55-70 (2017)*

Extracellular vesicles, such as exosomes and microvesicles, have been shown to contain potential microRNA (miRNA) biomarkers that may be utilized in the diagnosis of various diseases from cancer to neurological disorders. The unique nature of the extracellular vesicle bilayer allows miRNA to be protected from degradation making it an ideal source of material for biomarkers discovery from both fresh and archived samples. Here we describe the quantitative analysis of miRNA isolated from exosomes by quantitative PCR and digital PCR.

3.2704 Synthesis and characterization of a novel rhodamine labeled cholesterol reporter
Maiwald, A., Bauer, O. and Gimpl, G.
Biochim. Biophys. Acta, ***1859***, *1099-1113 (2017)*

We introduce the novel fluorescent cholesterol probe RChol in which a sulforhodamine group is linked to the sixth carbon atom of the steroid backbone of cholesterol. The same position has recently been selected to generate the fluorescent reporter 6-dansyl-cholestanol (DChol) and the photoreactive 6-azi-cholestanol. In comparison with DChol, RChol is brighter, much more photostable, and requires less energy for excitation, i.e. favorable conditions for microscopical imaging. RChol easily incorporates into methyl-β-cyclodextrin forming a water-soluble inclusion complex that acts as an efficient sterol donor for cells and membranes. Like cholesterol, RChol possesses a free 3′OH group, a prerequisite to undergo intracellular esterification. RChol was also able to support the growth of cholesterol auxotrophic cells and can therefore substitute for cholesterol as a major component of the plasma membrane. According to subcellular fractionation, slight amounts of RChol (~ 12%) were determined in low-density Triton-insoluble fractions whereas the majority of RChol was localized in non-rafts fractions. In phase-separated giant unilamellar vesicles, RChol preferentially partitions in liquid-disordered membrane domains. Intracellular RChol was transferred to extracellular sterol acceptors such as high density lipoproteins in a dose-dependent manner. Unlike DChol, RChol was not delivered to the cholesterol storage pathway. Instead, it translocated to endosomes/lysosomes with some transient contacts to peroxisomes. Thus, RChol is considered as a useful probe to study the endosomal/lysosomal pathway of cholesterol.

3.2705 Achieving the Promise of Therapeutic Extracellular Vesicles: The Devil is in Details of Therapeutic Loading

Sutaria, D.S., Badawi, M., Phelps, M.A. and Schmittgen, T.D.
Pharm. Res., ***34(5)****, 1053-1066 (2017)*

Extracellular vesicles (EVs) represent a class of cell secreted organelles which naturally contain biomolecular cargo such as miRNA, mRNA and proteins. EVs mediate intercellular communication, enabling the transfer of functional nucleic acids from the cell of origin to the recipient cells. In addition, EVs make an attractive delivery vehicle for therapeutics owing to their increased stability in circulation, biocompatibility, low immunogenicity and toxicity profiles. EVs can also be engineered to display targeting moieties on their surfaces which enables targeting to desired tissues, organs or cells. While much has been learned on the role of EVs as cell communicators, the field of therapeutic EV application is currently under development. Critical to the future success of EV delivery system is the description of methods by which therapeutics can be successfully and efficiently loaded within the EVs. Two methods of loading of EVs with therapeutic cargo exist, endogenous and exogenous loading. We have therefore focused this review on describing the various published approaches for loading EVs with therapeutics.

3.2706 Exosome and Microvesicle-Enriched Fractions Isolated from Mesenchymal Stem Cells by Gradient Separation Showed Different Molecular Signatures and Functions on Renal Tubular Epithelial Cells

Collino, F., Pomatto, M., Bruno, S., Lindoso, R.S., Tapparo, M., Sicheng, W., Quesenberry, P. and Camussi, G.
Stem Cell Rev. and Rep., ***13(2)****, 226-243 (2017)*

Several studies have suggested that extracellular vesicles (EVs) released from mesenchymal stem cells (MSCs) may mediate MSC paracrine action on kidney regeneration. This activity has been, at least in part, ascribed to the transfer of proteins/transcription factors and different RNA species. Information on the RNA/protein content of different MSC EV subpopulations and the correlation with their biological activity is currently incomplete. The aim of this study was to evaluate the molecular composition and the functional properties on renal target cells of MSC EV sub-populations separated by gradient floatation. The results demonstrated heterogeneity in quantity and composition of MSC EVs. Two peaks of diameter were observed (90–110 and 170–190 nm). The distribution of exosomal markers and miRNAs evaluated in the twelve gradient fractions showed an enrichment in fractions with a flotation density of 1.08–1.14 g/mL. Based on this observation, we evaluated the biological activity on renal cell proliferation and apoptosis resistance of low (CF1), medium (CF2) and high (CF3) floatation density fractions. EVs derived from all fractions, were internalized by renal cells, CF1 and CF2 but not CF3 fraction stimulated significant cell proliferation. CF2 also inhibited apoptosis on renal tubular cells submitted to ischemia-reperfusion injury. Comparative miRNomic and proteomic profiles reveal a cluster of miRNAs and proteins common to all three fractions and an enrichment of selected molecules related to renal regeneration in CF2 fraction. In conclusion, the CF2 fraction enriched in exosomal markers was the most active on renal tubular cell proliferation and protection from apoptosis.

3.2707 Defining the dynamin-based ring organizing center on the peroxisome-dividing machinery isolated from Cyanidioschyzon merolae

Imoto, Y., Abe, Y., Okumoto, K., Honsbo, M., Kuroiwa, H., Kuroiwa, T. and Fujiki, Y.
J. Cell Sci., ***130(5)****, 853-867 (2017)*

Organelle division is executed through contraction of a ring-shaped supramolecular dividing machinery. A core component of the machinery is the dynamin-based ring conserved during the division of mitochondrion, plastid and peroxisome. Here, using isolated peroxisome-dividing (POD) machinery from a unicellular red algae, *Cyanidioschyzon merolae*, we identified a dynamin-based ring organizing center (DOC) that acts as an initiation point for formation of the dynamin-based ring. *C. merolae* contains a single peroxisome, the division of which can be highly synchronized by light–dark stimulation; thus, intact POD machinery can be isolated in bulk. Dynamin-based ring homeostasis is maintained by the turnover of the GTP-bound form of the dynamin-related protein Dnm1 between the cytosol and division machinery via the DOC. A single DOC is formed on the POD machinery with a diameter of 500–700 nm, and the dynamin-based ring is unidirectionally elongated from the DOC in a manner that is dependent on GTP concentration. During the later step of membrane fission, the second DOC is formed and constructs the double dynamin-based ring to make the machinery thicker. These findings provide new insights to define fundamental mechanisms underlying the dynamin-based membrane fission in eukaryotic cells.

3.2708 The VDAC2–BAK axis regulates peroxisomal membrane permeability
Hosoi, K-i., Miyata, N., Mukai, S., Furuki, S., Okumoto, K., Cheng, E.H. and Fujiki, Y.
J. Cell Biol., ***216(3)***, *709-721 (2017)*

Peroxisomal biogenesis disorders (PBDs) are fatal genetic diseases consisting of 14 complementation groups (CGs). We previously isolated a peroxisome-deficient Chinese hamster ovary cell mutant, ZP114, which belongs to none of these CGs. Using a functional screening strategy, VDAC2 was identified as rescuing the peroxisomal deficiency of ZP114 where VDAC2 expression was not detected. Interestingly, knockdown of *BAK* or overexpression of the BAK inhibitors BCL-X_L and MCL-1 restored peroxisomal biogenesis in ZP114 cells. Although VDAC2 is not localized to the peroxisome, loss of VDAC2 shifts the localization of BAK from mitochondria to peroxisomes, resulting in peroxisomal deficiency. Introduction of peroxisome-targeted BAK harboring the Pex26p transmembrane region into wild-type cells resulted in the release of peroxisomal matrix proteins to cytosol. Moreover, overexpression of BAK activators PUMA and BIM permeabilized peroxisomes in a BAK-dependent manner. Collectively, these findings suggest that BAK plays a role in peroxisomal permeability, similar to mitochondrial outer membrane permeabilization.

3.2709 Chromatin remodeling during in vivo neural stem cells differentiating to neurons in early Drosophila embryos
Ye, Y., Li, M., Gu, L., Chen, X., Shi, J., Zhang, X. and Jiang, C.
Cell Death and Differentiation, ***24(3)***, *409-420 (2017)*

Neurons are a key component of the nervous system and differentiate from multipotent neural stem cells (NSCs). Chromatin remodeling has a critical role in the differentiation process. However, its *in vivo* epigenetic regulatory role remains unknown. We show here that nucleosome depletion regions (NDRs) form in both proximal promoters and distal enhancers during NSCs differentiating into neurons in the early *Drosophila* embryonic development. NDR formation in the regulatory regions involves nucleosome shift and eviction. Nucleosome occupancy in promoter NDRs is inversely proportional to the gene activity. Genes with promoter NDR formation during differentiation are enriched for functions related to neuron development and maturation. Active histone-modification signals (H3K4me3 and H3K9ac) in promoters are gained in neurons in two modes: *de novo* establishment to high levels or increase from the existing levels in NSCs. The gene sets corresponding to the two modes have different neuron-related functions. Dynamic changes of H3K27ac and H3K9ac signals in enhancers and promoters synergistically repress genes associated with neural stem or progenitor cell-related pluripotency and upregulate genes associated with neuron projection morphogenesis, neuron differentiation, and so on. Our results offer new insights into chromatin remodeling during *in vivo* neuron development and lay a foundation for its epigenetic regulatory mechanism study of other lineage specification.

3.2710 Daclatasvir Prevents Hepatitis C Virus Infectivity by Blocking Transfer of the Viral Genome to Assembly Sites
Boson, B., Denolly, S., Turlure, F., Chamot, C., Dreux, M. and Cosset, F-L.
Gastroenterol., ***152(4)***, *895-907 (2017)*

Background & Aims
Daclatasvir is a direct-acting antiviral agent and potent inhibitor of NS5A, which is involved in replication of the hepatitis C virus (HCV) genome, presumably via membranous web shaping, and assembly of new virions, likely via transfer of the HCV RNA genome to viral particle assembly sites. Daclatasvir inhibits the formation of new membranous web structures and, ultimately, of replication complex vesicles, but also inhibits an early assembly step. We investigated the relationship between daclatasvir-induced clustering of HCV proteins, intracellular localization of viral RNAs, and inhibition of viral particle assembly.
Methods
Cell-culture–derived HCV particles were produced from Huh7.5 hepatocarcinoma cells in presence of daclatasvir for short time periods. Infectivity and production of physical particles were quantified and producer cells were subjected to subcellular fractionation. Intracellular colocalization between core, E2, NS5A, NS4B proteins, and viral RNAs was quantitatively analyzed by confocal microscopy and by structured illumination microscopy.
Results
Short exposure of HCV-infected cells to daclatasvir reduced viral assembly and induced clustering of structural proteins with non-structural HCV proteins, including core, E2, NS4B, and NS5A. These clustered structures appeared to be inactive assembly platforms, likely owing to loss of functional

connection with replication complexes. Daclatasvir greatly reduced delivery of viral genomes to these core clusters without altering HCV RNA colocalization with NS5A. In contrast, daclatasvir neither induced clustered structures nor inhibited HCV assembly in cells infected with a daclatasvir-resistant mutant (NS5A-Y93H), indicating that daclatasvir targets a mutual, specific function of NS5A inhibiting both processes.
Conclusions
In addition to inhibiting replication complex biogenesis, daclatasvir prevents viral assembly by blocking transfer of the viral genome to assembly sites. This leads to clustering of HCV proteins because viral particles and replication complex vesicles cannot form or egress. This dual mode of action of daclatasvir could explain its efficacy in blocking HCV replication in cultured cells and in treatment of patients with HCV infection.

3.2711 LC–MS/MS Based Quantitation of ABC and SLC Transporter Proteins in Plasma Membranes of Cultured Primary Human Retinal Pigment Epithelium Cells and Immortalized ARPE19 Cell Line
Mol. Pharmeceut., 14(3), 605-613 (2017)

The retinal pigment epithelium (RPE) forms the outer blood–retinal barrier between neural retina and choroid. The RPE has several important vision supporting functions, such as transport mechanisms that may also modify pharmacokinetics in the posterior eye segment. Expression of plasma membrane transporters in the RPE cells has not been quantitated. The aim of this study was to characterize and compare transporter protein expression in the ARPE19 cell line and hfRPE (human fetal RPE) cells by using quantitative targeted absolute proteomics (QTAP). Among 41 studied transporters, 16 proteins were expressed in hfRPE and 13 in ARPE19 cells. MRP1, MRP5, GLUT1, 4F2hc, TAUT, CAT1, LAT1, and MATE1 proteins were detected in both cell lines within 4-fold differences. MPR7, OAT2 and RFC1 were detected in the hfRPE cells, but their expression levels were below the limit of quantification in ARPE19 cells. PCFT was detected in both studied cell lines, but the expression was over 4-fold higher in hfRPE cells. MCT1, MCT4, MRP4, and Na+/K+ ATPase were upregulated in the ARPE19 cell line showing over 4-fold differences in the quantitative expression values. Expression levels of 25 transporters were below the limit of quantification in both cell models. In conclusion, we present the first systematic and quantitative study on transporter protein expression in the plasma membranes of ARPE19 and hfRPE cells. Overall, transporter expression in the ARPE19 and hfRPE cells correlated well and the absolute expression levels were similar, but not identical. The presented quantitative expression levels could be a useful basis for further studies on drug permeation in the outer blood–retinal barrier.

3.2712 Immunoactive Clostridial Membrane Vesicle Production Is Regulated by a Sporulation Factor
Obana, N., Nakao, R., Nagayama, K., nakamura, K., Senpuku, H. and Nomura, N.
*Infect. Immun., **85(5)**, e00096-17 (2017)*

Recently, many Gram-positive bacteria as well as Gram-negative bacteria have been reported to produce membrane vesicles (MVs), but little is known regarding the regulators involved in MV formation. We found that a Gram-positive anaerobic pathogen, Clostridium perfringens, produces MVs predominantly containing membrane proteins and cell wall components. These MVs stimulated proinflammatory cytokine production in mouse macrophage-like cells. We suggested that MVs induced interleukin-6 production through the Toll-like receptor 2 (TLR2) signaling pathway. Thus, the MV could have a role in the bacterium-host interaction and bacterial infection pathogenesis. Moreover, we found that the sporulation master regulator gene spo0A was required for vesiculogenesis. A conserved, phosphorylated aspartate residue of Spo0A was indispensable for MV production, suggesting that the phosphorylation of Spo0A triggers MV production. Multiple orphan sensor kinases necessary for sporulation were also required to maximize MV production. These findings imply that C. perfringens actively produces immunoactive MVs in response to the environment changing, as recognized by membrane-spanning sensor kinases and by modulating the phosphorylation level of Spo0A.

3.2713 Predicting the targeting of tail-anchored proteins to subcellular compartments in mammalian cells
Costello, J.L. et al
*J. Cell Sci., **130**, 1675-1687 (2017)*

Tail-anchored (TA) proteins contain a single transmembrane domain (TMD) at the C-terminus that anchors them to the membranes of organelles where they mediate critical cellular processes. Accordingly, mutations in genes encoding TA proteins have been identified in a number of severe inherited disorders. Despite the importance of correctly targeting a TA protein to its appropriate membrane, the mechanisms

and signals involved are not fully understood. In this study, we identify additional peroxisomal TA proteins, discover more proteins that are present on multiple organelles, and reveal that a combination of TMD hydrophobicity and tail charge determines targeting to distinct organelle locations in mammals. Specifically, an increase in tail charge can override a hydrophobic TMD signal and re-direct a protein from the ER to peroxisomes or mitochondria and vice versa. We show that subtle changes in those parameters can shift TA proteins between organelles, explaining why peroxisomes and mitochondria have many of the same TA proteins. This enabled us to associate characteristic physicochemical parameters in TA proteins with particular organelle groups. Using this classification allowed successful prediction of the location of uncharacterized TA proteins for the first time.

3.2714 Localization of the placental BCRP/ABCG2 transporter to lipid rafts: Role for cholesterol in mediating efflux activity

Szilagyi, J.T., Vetrano, A.M., Laskin, J.D. and Aleksunes, L.M.
*Placenta, **55**, 29-36 (2017)*

Introduction
The breast cancer resistance protein (BCRP/*ABCG2*) is an efflux transporter in the placental barrier. By transporting chemicals from the fetal to the maternal circulation, BCRP limits fetal exposure to a range of drugs, toxicants, and endobiotics such as bile acids and hormones. The purpose of the present studies was to 1) determine whether BCRP localizes to highly-ordered, cholesterol-rich lipid raft microdomains in placenta microvillous membranes, and 2) determine the impact of cholesterol on BCRP-mediated placental transport *in vitro*.
Methods
BCRP expression was analyzed in lipid rafts isolated from placentas from healthy, term pregnancies and BeWo trophoblasts by density gradient ultracentrifugation. BeWo cells were also tested for their ability to efflux BCRP substrates after treatment with the cholesterol sequestrant methyl-β-cyclodextrin (MβCD, 5 mM, 1 h) or the cholesterol synthesis inhibitor pravastatin (200 μM, 48 h).
Results and discussion
BCRP was found to co-localize with lipid raft proteins in detergent-resistant, lipid raft-containing fractions from placental microvillous membranes and BeWo cells. Treatment of BeWo cells with MβCD redistributed BCRP protein into higher density non-lipid raft fractions. Repletion of the cells with cholesterol restored BCRP localization to lipid raft-containing fractions. Treatment of BeWo cells with MβCD or pravastatin increased cellular retention of two BCRP substrates, the fluorescent dye Hoechst 33342 and the mycotoxin zearalenone. Repletion with cholesterol restored BCRP transporter activity. Taken together, these data demonstrate that cholesterol may play a critical role in the post-translational regulation of BCRP in placental lipid rafts.

3.2715 Helicobacter pylori-derived extracellular vesicles increased in the gastric juices of gastric adenocarcinoma patients and induced inflammation mainly via specific targeting of gastric epithelial cells

Choi, H-I., Choi, J-P., Seo, J., Kim, B.J., Rho, M., Han, J.K. and Kim, J.G.
*Exp. Mol. Med., **49**, e330 (2017)*

Evidence indicates that *Helicobacter pylori* is the causative agent of chronic gastritis and perhaps gastric malignancy. Extracellular vesicles (EVs) play an important role in the evolutional process of malignancy due to their genetic material cargo. We aimed to evaluate the clinical significance and biological mechanism of *H. pylori* EVs on the pathogenesis of gastric malignancy. We performed 16S rDNA-based metagenomic analysis of gastric juices either from endoscopic or surgical patients. From each sample of gastric juices, the bacteria and EVs were isolated. We evaluated the role of *H. pylori* EVs on the development of gastric inflammation *in vitro* and *in vivo*. IVIS spectrum and confocal microscopy were used to examine the distribution of EVs. The metagenomic analyses of the bacteria and EVs showed that *Helicobacter* and *Streptococcus* are the two major bacterial genera, and they were significantly increased in abundance in gastric cancer (GC) patients. *H. pylori* EVs are spherical and contain CagA and VacA. They can induce the production of tumor necrosis factor-α, interleukin (IL)-6 and IL-1β by macrophages, and IL-8 by gastric epithelial cells. Also, EVs induce the expression of interferon gamma, IL-17 and EV-specific immunoglobulin Gs *in vivo* in mice. EVs were shown to infiltrate and remain in the mouse stomach for an extended time. *H. pylori* EVs, which are abundant in the gastric juices of GC patients, can induce inflammation and possibly cancer in the stomach, mainly via the production of inflammatory mediators from gastric epithelial cells after selective uptake by the cells.

3.2716 Visualization of a Mammalian Mitochondrion by Coherent X-ray Diffractive Imaging
Kim, Y., Kim, C., Kwon, O.Y., Nam, D., Kim, S.S., Park, J.H., Kim, S., Gallagher-Jones,. M., Kohmura, Y., Ishikawa, T., Song, C., Tae, G. and Noh, D.Y.
*Scientific Reports, **7**:1850 (2017)*

We report a three dimensional (3D) quantitative visualization of a mammalian mitochondrion by coherent x-ray diffractive imaging (CXDI) using synchrotron radiation. The internal structures of a mitochondrion from a mouse embryonic fibroblast cell line (NIH3T3) were visualized by tomographic imaging at approximately 60 nm resolution without the need for sectioning or staining. The overall structure consisted of a high electron density region, composed of the outer and inner membranes and the cristae cluster, which enclosed the lower density mitochondrial matrix. The average mass density of the mitochondrion was about 1.36 g/cm3. Sectioned images of the cristae reveal that they have neither a baffle nor septa shape but were instead irregular. In addition, a high resolution, about 14 nm, 2D projection image was captured of a similar mitochondrion with the aid of strongly scattering Au reference objects. Obtaining 3D images at this improved resolution will allow CXDI to be an effective and nondestructive method for investigating the innate structure of mitochondria and other important life supporting organelles.

3.2717 SorLA in Interleukin-6 Signaling and Turnover
Larsen, J.V. and Petersen, C.M.
*Mol. Cell. Biol., **37(11)**, e00641 (2017)*

Interleukin-6 (IL-6) is a multifunctional cytokine with important functions in various physiologic processes. Mice lacking IL-6 exhibit multiple phenotypic abnormalities, such as an inadequate immune and acute-phase response, and elevated levels of circulating IL-6 have been found to accompany several pathological conditions. IL-6 binds the nonsignaling IL-6 receptor (IL-6R), which is expressed as a transmembrane, as well as a secreted circulating protein, before it engages homodimeric gp130 for signaling. Complex formation between IL-6 and the membrane-bound IL-6 receptor gives rise to classic *cis* signaling, whereas complex formation between IL-6 and the soluble IL-6R results in *trans* signaling. Here, we report that the endocytic receptor SorLA targets IL-6 and IL-6R. We present evidence that SorLA mediates efficient cellular uptake of both IL-6 and the circulating IL-6R in astrocytes. We further show that SorLA interacts with the membrane-bound IL-6R at the cell surface and thereby downregulates IL-6 *cis* signaling. Finally, we find that the SorLA ectodomain, released from the cell membrane upon enzymatic cleavage of full-length SorLA, may act as an IL-6 carrier protein that stabilizes IL-6 and its capacity for *trans* signaling. Interleukin-6 (IL-6) is a multifunctional cytokine with important functions in various physiologic processes. Mice lacking IL-6 exhibit multiple phenotypic abnormalities, such as an inadequate immune and acute-phase response, and elevated levels of circulating IL-6 have been found to accompany several pathological conditions. IL-6 binds the nonsignaling IL-6 receptor (IL-6R), which is expressed as a transmembrane, as well as a secreted circulating protein, before it engages homodimeric gp130 for signaling. Complex formation between IL-6 and the membrane-bound IL-6 receptor gives rise to classic cis signaling, whereas complex formation between IL-6 and the soluble IL-6R results in trans signaling. Here, we report that the endocytic receptor SorLA targets IL-6 and IL-6R. We present evidence that SorLA mediates efficient cellular uptake of both IL-6 and the circulating IL-6R in astrocytes. We further show that SorLA interacts with the membrane-bound IL-6R at the cell surface and thereby downregulates IL-6 cis signaling. Finally, we find that the SorLA ectodomain, released from the cell membrane upon enzymatic cleavage of full-length SorLA, may act as an IL-6 carrier protein that stabilizes IL-6 and its capacity for trans signaling.

3.2718 Gamma-glutamyltransferase activity in exosomes as a potential marker for prostate cancer
Kawakami, K., Fujita, Y., matsuda, Y., Arai, T., Horie, K., kameyama, K., Kato, T., Masunaga, K., Kasuya, Y., Tanaka, M., Mizutani, K., Deguchi, T. and Ito, M.
*BMC Cancer, **17**:316 (2017)*

Background
Exosomes or extracellular vesicles have the potential as a diagnostic marker for various diseases including cancer. In order to identify novel exosomal markers for prostate cancer (PC), we performed proteomic analysis of exosomes isolated from PC cell lines and examined the usefulness of the marker in patients.
Methods
Exosomes isolated by differential centrifugation from the culture medium of androgen-dependent LNCaP prostate cancer cell line and its sublines of partially androgen-independent C4, androgen-independent C4–2 and bone metastatic C4–2B were subjected to iTRAQ-based proteomic analysis. Exosomes were also

isolated by immunocapture and separated by size exclusion chromatography and density gradient centrifugation. Protein expression was determined by Western blot analysis. GGT activity was measured using a fluorescent probe, γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG). Immunohistochemical analysis of tissues was performed using anti-GGT1 antibody.
Results
Among proteins upregulated in C4–2 and C4–2B cells than in LNCaP cells, we focused on gamma-glutamyltransferase 1 (GGT1), a cell-surface enzyme that regulates the catabolism of extracellular glutathione. The levels of both GGT1 large and small subunits were elevated in exosomes isolated from C4–2 and C4–2B cells by differential centrifugation and by immunocapture with anti-CD9 or -prostate-specific membrane antigen (PSMA) antibody. In cell lysates and exosomes, GGT1 expression correlated with GGT activity. Size exclusion chromatography of human serum demonstrated the presence of GGT activity and GGT1 subunits in fractions positive for CD9. Density gradient centrifugation revealed the co-presence of GGT1 subunits with CD9 in exosomes isolated by differential centrifugation from human serum. Since GGT activity correlated with GGT1 expression in serum exosomes isolated by differential centrifugation, we measured serum exosomal GGT activity in patients. Unexpectedly, we found that serum exosomal GGT activity was significantly higher in PC patients than in benign prostatic hyperplasia (BPH) patients. In support of this finding, immunohistochemical analysis showed increased GGT1 expression in PC tissues compared with BPH tissues.
Conclusions
Our results suggest that serum exosomal GGT activity could be a useful biomarker for PC.

3.2719 **Review: Bio-compartmentalization of microRNAs in exosomes during gestational diabetes mellitus**
Iljas, J.D., Guanzon, D., Elfeky, O., Rice, G.E. and Solomon, C.
*Placenta, **54**, 76-82 (2017)*

Analysis of the human genome revealed that only 1.2% encoded for proteins, which raised questions regarding the biological significance of the remaining genome. We now know that approximately 80% of the genome serves at least one biochemical function within the cell. A portion of this 80% consists of a family of non-coding regulatory RNAs, one important member being microRNAs (miRNAs). miRNAs can be detected in tissues and biofluids, where miRNAs in the latter can be bound to proteins or encapsulated within lipid vesicles such as exosomes. Gestational diabetes mellitus (GDM) is a complication of pregnancy, which has harmful health impacts on both the fetus as well as the mother. The incidence of GDM worldwide varies, but reached 18% in the HAPO cohort using the new International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria. Not only has GDM been associated with increased risks of further complications during pregnancy, but also poses long-term risks for both the mother and the baby. Thus, understanding the pathophysiology of GDM is important from a public health perspective. Literature has demonstrated that GDM is associated with elevated levels of circulating exosomes in maternal circulation. However, there is a paucity of data defining the expression, role, and diagnostic utility of miRNAs in GDM. This review briefly summarizes recent advances in the function and quantification of intracellular and extracellular miRNAs in GDM.

3.2720 **Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research**
Vergauwen, G., Dhondt, B., Van Deun, J., De Smedt, E., Berx, G., Timmerman, E., Gevaert, K., Miinalainen, I., Cocquyt, V., Braems, G., Ven den Broecke, R., Denys, H., De Wevwer, O. and Hendrix, A.
*Scientific Reports, **7**:2704 (2017)*

Identification and validation of extracellular vesicle (EV)-associated biomarkers requires robust isolation and characterization protocols. We assessed the impact of some commonly implemented pre-analytical, analytical and post-analytical variables in EV research. Centrifugal filters with different membrane types and pore sizes are used to reduce large volume biofluids prior to EV isolation or to concentrate EVs. We compared five commonly reported filters for their efficiency when using plasma, urine and EV-spiked PBS. Regenerated cellulose membranes with pore size of 10 kDa recovered EVs the most efficient. Less than 40% recovery was achieved with other filters. Next, we analyzed the effect of the type of protein assays to measure EV protein in colorimetric and fluorometric kits. The fluorometric assay Qubit measured low concentration EV and BSA samples the most accurately with the lowest variation among technical and biological replicates. Lastly, we quantified Optiprep remnants in EV samples from density gradient ultracentrifugation and demonstrate that size-exclusion chromatography efficiently removes Optiprep from EVs. In conclusion, choice of centrifugal filters and protein assays confound EV analysis and should be

carefully considered to increase efficiency towards biomarker discovery. SEC-based removal of Optiprep remnants from EVs can be considered for downstream applications.

3.2721 Prions on the run: How extracellular vesicles serve as delivery vehicles for self-templating protein aggregates

Liu, S., Hossinger, A., Göbbels, S. and Vorberg, I.M.
*Prion, **11**, 98-112 (2017)*

Extracellular vesicles (EVs) are actively secreted, membrane-bound communication vehicles that exchange biomolecules between cells. EVs also serve as dissemination vehicles for pathogens, including prions, proteinaceous infectious agents that cause transmissible spongiform encephalopathies (TSEs) in mammals. Increasing evidence accumulates that diverse protein aggregates associated with common neurodegenerative diseases are packaged into EVs as well. Vesicle-mediated intercellular transmission of protein aggregates can induce aggregation of homotypic proteins in acceptor cells and might thereby contribute to disease progression. Our knowledge of how protein aggregates are sorted into EVs and how these vesicles adhere to and fuse with target cells is limited. Here we review how TSE prions exploit EVs for intercellular transmission and compare this to the transmission behavior of self-templating cytosolic protein aggregates derived from the yeast prion domain Sup 35 NM. Artificial NM prions are non-toxic to mammalian cell cultures and do not cause loss-of-function phenotypes. Importantly, NM particles are also secreted in association with exosomes that horizontally transmit the prion phenotype to naive bystander cells, a process that can be monitored with high accuracy by automated high throughput confocal microscopy. The high abundance of mammalian proteins with amino acid stretches compositionally similar to yeast prion domains makes the NM cell model an attractive model to study self-templating and dissemination properties of proteins with prion-like domains in the mammalian context.

3.2722 Isolation of membrane vesicles from prokaryotes: a technical and biological comparison reveals heterogeneity

Singorenko, P.D., Chang, V., Whitcombe, A., Simonov, D., Hong, J., Phillips, A., Swift, S. and Blenkiron, C.
*J. Extracellular Vesicles, **6**, 1324731 (2017)*

Prokaryotes release membrane vesicles (MVs) with direct roles in disease pathogenesis. MVs are heterogeneous when isolated from bacterial cultures so Density Gradient Centrifugation (DGC) is valuable for separation of MV subgroups from contaminating material. Here we report the technical variability and natural biological heterogeneity seen between DGC preparations of MVs for *Mycobacterium smegmatis* and *Escherichia coli* and compare these DGC data with size exclusion chromatography (SEC) columns. Crude preparations of MVs, isolated from cultures by ultrafiltration and ultracentrifugation were separated by DGC with fractions manually collected as guided by visible bands. Yields of protein, RNA and endotoxin, protein banding and particle counts were analysed in these. DGC and SEC methods enabled separation of molecularly distinct MV populations from crude MVs. DGC banding profiles were unique for each of the two species of bacteria tested and further altered by changing culture conditions, for example with iron supplementation. SEC is time efficient, reproducible and cost effective method that may also allow partial LPS removal from Gram-negative bacterial MVs. In summary, both DGC and SEC are suitable for the separation of mixed populations of MVs and we advise trials of both, coupled with complete molecular and single vesicle characterisation prior to downstream experimentation.

3.2723 High-throughput sequencing of two populations of extracellular vesicles provides an mRNA signature that can be detected in the circulation of breast cancer patients

Conley, A., Minciacchi, V.R., Lee, D.H., Knudsen, B.S., karlan, B.Y., Citrigno, L., Viglietto, G., Tewari, M., Freeman, M.R., Demichelis, F. and Di Vizio, D.
*RNA Biology, **14(3)**, 305-316 (2017)*

Extracellular vesicles (EVs) contain a wide range of RNA types with a reported prevalence of non-coding RNA. To date a comprehensive characterization of the protein coding transcripts in EVs is still lacking. We performed RNA-Sequencing (RNA-Seq) of 2 EV populations and identified a small fraction of transcripts that were expressed at significantly different levels in large oncosomes and exosomes, suggesting they may mediate specialized functions. However, these 2 EV populations exhibited a common mRNA signature that, in comparison to their donor cells, was significantly enriched in mRNAs encoding E2F transcriptional targets and histone proteins. These mRNAs are primarily expressed in the S-phase of the cell cycle, suggesting that they may be packaged into EVs during S-phase. *In silico* analysis using

subcellular compartment transcriptome data from the ENCODE cell line compendium revealed that EV mRNAs originate from a cytoplasmic RNA pool. The EV signature was independently identified in plasma of patients with breast cancer by RNA-Seq. Furthermore, several transcripts differentially expressed in EVs from patients versus controls mirrored differential expression between normal and breast cancer tissues. Altogether, this largest high-throughput profiling of EV mRNA demonstrates that EVs carry tumor-specific alterations and can be interrogated as a source of cancer-derived cargo.

3.2724 **Unique molecular profile of exosomes derived from primary human proximal tubular epithelial cells under diseased conditions**
Wang, X., Wilkinson, R., Kildey, K., Potriquet, J., Mulvenna, J., Lobb, R.J., Möller, A., Cloonan, N., Mukhopadhyay, P., Kassianos, A.J. and Healy, H.
J. Extracellular Vesicles, ***6(1)****, 1314073 (2017)*

Human proximal tubular epithelial cells (PTEC) of the kidney are known to respond to and mediate the disease process in a wide range of kidney diseases, yet their exosomal production and exosome molecular cargo remain a mystery. Here we investigate, for the first time, the production and molecular content of exosomes derived from primary human PTEC cultured under normal and diseased conditions representing a spectrum of *in vivo* disease severity from early inflammation, experienced in multiple initial kidney disease states, through to hypoxia, frequently seen in late stage chronic kidney disease (CKD) due to fibrosis and vascular compromise. We demonstrate a rapid reproducible methodology for the purification of PTEC-derived exosomes, identify increased numbers of exosomes from disease-state cultures and identify differential expression levels of both known and unique miRNA and protein species from exosomes derived from different disease-culture conditions. The validity of our approach is supported by the identification of miRNA, proteins and pathways with known CKD associations, providing a rationale to further evaluate these novel and known pathways as targets for therapeutic intervention.

3.2725 **Phosphatidylserine synthesis at membrane contact sites promotes its transport out of the ER**
Kannan, M., Lahiri, S., Liu, L-K., Choudhary, V. and Prinz, W.A.
J. Lipid Res., ***58(3)****, 553-562 (2017)*

Close contacts between organelles, often called membrane contact sites (MCSs), are regions where lipids are exchanged between organelles. Here, we identify a novel mechanism by which cells promote phospholipid exchange at MCSs. Previous studies have shown that phosphatidylserine (PS) synthase activity is highly enriched in portions of the endoplasmic reticulum (ER) in contact with mitochondria. The objective of this study was to determine whether this enrichment promotes PS transport out of the ER. We found that PS transport to mitochondria was more efficient when PS synthase was fused to a protein in the ER at ER-mitochondria contacts than when it was fused to a protein in all portions of the ER. Inefficient PS transport to mitochondria was corrected by increasing tethering between these organelles. PS transport to endosomes was similarly enhanced by PS production in regions of the ER in contact with endosomes. Together, these findings indicate that PS production at MCSs promotes PS transport out of the ER and suggest that phospholipid production at MCSs may be a general mechanism of channeling lipids to specific cellular compartments.

3.2726 **γ2 and γ1AP-1 complexes: Different essential functions and regulatory mechanisms in clathrin-dependent protein sorting**
Zizioli, D., Geumann, C., Kratzke, M., Mishra, R., Borsani, G., Finazzi, D., Candiello, E. and Schu, P.
Eur. J. Cell Biol., ***96****, 356-368 (2017)*

γ2 adaptin is homologous to γ1, but is only expressed in vertebrates while γ1 is found in all eukaryotes. We know little about γ2 functions and their relation to γ1. γ1 is an adaptin of the heterotetrameric AP-1 complexes, which sort proteins in and do form clathrin-coated transport vesicles and they also regulate maturation of early endosomes. γ1 knockout mice develop only to blastocysts and thus γ2 does not compensate γ1-deficiency in development. γ2 has not been classified as a clathrin-coated vesicle adaptor protein in proteome analyses and functions for monomeric γ2 in endosomal protein sorting have been proposed, but adaptin interaction studies suggested formation of heterotetrameric AP-1/γ2 complexes. We detected γ2 at the *trans*-Golgi network, on peripheral vesicles and identified γ2 clathrin-coated vesicles in mice. Ubiquitous σ1A and tissue-specific σ1B adaptins bind γ2 and γ1. σ1B knockout in mice does not effect γ1/σ1A AP-1 levels, but γ2/σ1A AP-1 levels are increased in brain and adipocytes. Also γ2 is

essential in development. In zebrafish AP-1/γ2 and AP-1/γ1 fulfill different, essential functions in brain and the vascular system.

3.2727 The role of extracellular vesicles in malaria biology and pathogenesis
Sampaio, N.G., Cheng, L. and Eriksson, E.M.
Malaria J., ***16****:245 (2017)*

In the past decade, research on the functions of extracellular vesicles in malaria has expanded dramatically. Investigations into the various vesicle types, from both host and parasite origin, has revealed important roles for extracellular vesicles in disease pathogenesis and susceptibility, as well as cell–cell communication and immune responses. Here, work relating to extracellular vesicles in malaria is reviewed, and the areas that remain unknown and require further investigations are highlighted.

3.2728 Exosomes in Cancer Nanomedicine and Immunotherapy: Prospects and Challenges
Syn, N.L., Wang, L., Chow, E.K-H., Lim, C.T. and Goh, B-C,
Trends in Biotechnology, ***35(7)****, 665-676 (2017)*

Exosomes (versatile, cell-derived nanovesicles naturally endowed with exquisite target-homing specificity and the ability to surmount *in vivo* biological barriers) hold substantial promise for developing exciting approaches in drug delivery and cancer immunotherapy. Specifically, bioengineered exosomes are being successfully deployed to deliver potent tumoricidal drugs (siRNAs and chemotherapeutic compounds) preferentially to cancer cells, while a new generation of exosome-based therapeutic cancer vaccines has produced enticing results in early-phase clinical trials. Here, we review the state-of-the-art technologies and protocols, and discuss the prospects and challenges for the clinical development of this emerging class of therapeutics.

3.2729 Measurement of Cholesterol Transfer from Lysosome to Peroxisome Using an In Vitro Reconstitution Assay
Luo, J., Liao, Y-C., Xiao, J. and Song, B-L.
Methods in Mol. Biol., ***1583****, 141-161 (2017)*

Low-density lipoproteins (LDLs) are taken up by the cell mainly through receptor-mediated endocytosis. LDL-derived cholesterol leaves lysosome and further transports to downstream organelles for specific cellular needs. We recently report that cholesterol transfers from lysosome to peroxisome through lysosome–peroxisome membrane contact (LPMC). Here, we use iodixanol density gradient centrifugation to isolate lysosomes and peroxisomes separately for the in vitro reconstitution of LPMC. We also apply ^{3}H-cholesterol-labeled lysosomes and peroxisomes in vitro to measure ^{3}H-cholesterol transfer through LPMC.

3.2730 Isolation of Peroxisomes from Rat Liver and Cultured Hepatoma Cells by Density Gradient Centrifugation
Manner, A. and Islinger, M.
Methods in Mol. Biol., ***1595****, 1-11 (2017)*

Subcellular fractionation is still a valuable technique to unravel organelle-specific proteomes, validate the location of uncharacterized proteins, or to functionally analyze import and metabolism in individual subcellular compartments. In this respect, density gradient centrifugation still represents a very classic, indispensable technique to isolate and analyze peroxisomes. Here, we present two independent protocols for the purification of peroxisomes from either liver tissue or the HepG2 hepatoma cell line. While the former permits the isolation of highly pure peroxisomes suitable for, e.g., subcellular proteomics experiments, the latter protocol yields peroxisomal fractions from considerably less purity but allows to easily modify metabolic conditions in the culture medium or to genetically manipulate the peroxisomal compartment. In this respect, both purification methods represent alternative tools to be applied in experiments investigating peroxisome physiology.

3.2731 Extracellular vesicle mimetics: Novel alternatives to extracellular vesicle-based theranostics, drug delivery, and vaccines
Kim, O.Y., Lee, J. and Gho, Y.S.
Seminars in Cell & Development Biol., ***67****, 74-82 (2017)*

Extracellular vesicles are nano-sized spherical bilayered proteolipids encasing various components. Cells of all domains of life actively release these vesicles to the surroundings including various biological fluids. These extracellular vesicles are known to play pivotal roles in numerous pathophysiological functions. Extracellular vesicles have distinct characteristics, like high biocompatibility, safety, and nano-sized diameters that allow efficient drug loading capacity and long blood circulation half-life. These characteristics of extracellular vesicles have engrossed many scientists to harness them as new tools for novel delivery systems. This review will highlight the current state of the arts and problems of such extracellular vesicle-based theranostics, drug delivery and vaccines, and introduce "extracellular vesicle mimetics" as the novel alternative of extracellular vesicles. We hope to provide insights into the potential of extracellular vesicle mimetics as superior substitute to the natural extracellular vesicles that can be applied to theranostics, drug delivery, and vaccines against various diseases.

3.2732 Selective inhibition of sterolO-acyltransferase 1 isozyme by beauveriolide III in intact cells
Ohshiro, T., Kobayashi, K., Ohba, M., Matsuda, D., Rudel, L.L., Takahashi, T., Doi, T. and Tomoda, H.
*Scientific Reports, **7**:4163 (2017)*

Beauveriolide III (BeauIII) inhibited sterol *O*-acyltransferases 1 and 2 (SOAT1 and SOAT2), which are endoplasmic reticulum (ER) membrane proteins, in an enzyme-based assay, and selectively inhibited SOAT1 in a cell-based assay using SOAT1-/SOAT2-CHO cells. This discrepancy in SOAT inhibition by BeauIII was investigated. In the enzyme-based assay, BeauIII inhibited SOAT1 and SOAT2 to a similar extent using microsomes prepared from cells disrupted under the strongest sonication condition. In semi-intact SOAT1-/SOAT2-CHO cells prepared by a treatment with digitonin (plasma membrane permeabilized), BeauIII selectively inhibited SOAT1 (IC_{50}; 5.0 μM (SOAT1) vs >90 μM (SOAT2)), while in those treated with saponin (plasma membrane and ER membrane permeabilized), BeauIII inhibited SOAT1 (IC_{50}, 1.8 μM) and SOAT2 (5.9 μM). SOAT1-selective inhibition by BeauIII was reproduced in intact ER fractions prepared from SOAT1/SOAT2-CHO cells. A Western blotting analysis revealed that biotin-labeled beauveriolide bound to the SOAT1 protein prepared from SOAT1-CHO cells. We concluded that BeauIII binds to a putative active site responsible for SOAT1 that is located on the cytosolic side of the ER, while BeauIII is not accessible to the corresponding active site for SOAT2 located on the luminal side.

3.2733 The role of exosomes in cancer metastasis
Steinbichler, T.B., Dudas, J., Riechelmann, H., Skvortsova, I-I.
*Seminars in Cancer Biol., **44**, 170-181 (2017)*

Exosomes are small membrane vesicles with a size ranging from 40 to 100 nm. They can serve as functional mediators in cell interaction leading to cancer metastasis. Metastasis is a complex multistep process of cancer cell invasion, survival in blood vessels, attachment to and colonization of the host organ. Exosomes influence every step of this cascade and can be targeted by oncological treatment. This review highlights the role of exosomes in the various steps of the metastatic cascade and how exosome dependent pathways can be targeted as therapeutic approach or used for liquid biopsies.

3.2734 Exosomes purified from a single cell type have diverse morphology
Zabeo, D., Cvjetkovic, A., Lässer, C., Schorb, M., L¨tvall, J. and Höög, J.L.
*J. Extracellular Vesicles, **6**:1329476 (2017)*

Extracellular vesicles (EVs) are produced by all known organisms and are important for cell communication and physiology. Great morphological diversity has been described regarding EVs found in body fluids such as blood plasma, breast milk, and ejaculate. However, a detailed morphological analysis has never been performed on exosomes when purified from a single cell type.
In this study we analysed and quantified, via multiple electron microscopy techniques, the morphology of exosomes purified from the human mast cell line HMC-1. The results revealed a wide diversity in exosome morphology, suggesting that subpopulations of exosomes with different and specific functions may exist. Our findings imply that a new, more efficient way of defining exosome subpopulations is necessary. A system was proposed where exosomes were classified into nine different categories according to their size and shape. Three additional morphological features were also found in exosomes regardless of their morphological classification.
These findings show that exosomes purified from a single cell line are also morphologically diverse, similar to previous observations for EVs in body fluids. This knowledge can help to improve the

interpretation of experimental results and widen our general understanding of the biological functions of exosomes.

3.2735 Synapsins regulate brain-derived neurotrophic factor-mediated synaptic potentiation and axon elongation by acting on membrane rafts
Kao, H-T., Ryoo, K., Lin, A., janoschka, S.R., Augustiine, G.J. and Porton, B.
Eur. J. Neurosci., ***45(8)***, *1085-1101 (2017)*

In neurons, intracellular membrane rafts are essential for specific actions of brain-derived neurotrophic factor (BDNF), which include the regulation of axon outgrowth, growth cone turning and synaptic transmission. Virtually, all the actions of BDNF are mediated by binding to its receptor, TrkB. The association of TrkB with the tyrosine kinase, Fyn, is critical for its localization to intracellular membrane rafts. Here, we show that synapsins, a family of highly amphipathic neuronal phosphoproteins, regulate membrane raft lipid composition and consequently, the ability of BDNF to regulate axon/neurite development and potentiate synaptic transmission. In the brains of mice lacking all synapsins, the expression of both BDNF and TrkB were increased, suggesting that BDNF/TrkB-mediated signaling is impaired. Consistent with this finding, synapsin-depleted neurons exhibit altered raft lipid composition, deficient targeting of Fyn to rafts, attenuated TrkB activation, and abrogation of BDNF-stimulated axon outgrowth and synaptic potentiation. Conversely, overexpression of synapsins in neuroblastoma cells results in corresponding reciprocal changes in raft lipid composition, increased localization of Fyn to rafts and promotion of BDNF-stimulated neurite formation. In the presence of synapsins, the ratio of cholesterol to estimated total phospholipids converged to 1, suggesting that synapsins act by regulating the ratio of lipids in intracellular membranes, thereby promoting lipid raft formation. These studies reveal a mechanistic link between BDNF and synapsins, impacting early development and synaptic transmission.

3.2736 Autophagosome formation is initiated at phosphatidylinositol synthase-enriched ER subdomains
Nishimura, T., Tamura, N., Kono, N., Shimanaka, Y., Arai, H., Yamamoto, H. and Mizushima, N.
EMBO J., ***36(12)***, *1719-1735 (2017)*

The autophagosome, a double-membrane structure mediating degradation of cytoplasmic materials by macroautophagy, is formed in close proximity to the endoplasmic reticulum (ER). However, how the ER membrane is involved in autophagy initiation and to which membrane structures the autophagy-initiation complex is localized have not been fully characterized. Here, we were able to biochemically analyze autophagic intermediate membranes and show that the autophagy-initiation complex containing ULK and FIP200 first associates with the ER membrane. To further characterize the ER subdomain, we screened phospholipid biosynthetic enzymes and found that the autophagy-initiation complex localizes to phosphatidylinositol synthase (PIS)-enriched ER subdomains. Then, the initiation complex translocates to the ATG9A-positive autophagosome precursors in a PI3P-dependent manner. Depletion of phosphatidylinositol (PI) by targeting bacterial PI-specific phospholipase C to the PIS domain impairs recruitment of downstream autophagy factors and autophagosome formation. These findings suggest that the autophagy-initiation complex, the PIS-enriched ER subdomain, and ATG9A vesicles together initiate autophagosome formation.

3.2737 Characterization of extracellular membrane vesicles of an Antarctic bacterium, Shewanella livingstonensis Ac10, and their enhanced production by alteration of phospholipid composition
Yokoyama, F., Kuwamoto, J., Imai, T. and Kurihara, T.
Extremophiles, ***21(4)***, *723-731 (2017)*

A cold-adapted bacterium, *Shewanella livingstonensis* Ac10, which produces eicosapentaenoic acid (EPA) as a component of its membrane phospholipids, is useful as a model to study the function of EPA and as a host for heterologous production of thermolabile proteins at low temperatures. In this study, we characterized extracellular membrane vesicles (EMVs) of this bacterium to examine the involvement of EPA in the biogenesis of EMVs and for the future application of EMVs to extracellular protein production. We found that this strain produced EMVs from the cell surface. Cryo-electron microscopic observation showed that the majority of the EMVs had a single-bilayer structure with an average diameter of 110 nm, though EMVs with double-bilayer membranes and other diverse structures were also observed. Quantitative analysis demonstrated that the EMV production was significantly increased (3–5 fold) by the depletion of EPA-containing phospholipids. The lack of EPA also altered the protein composition of EMVs. In particular, incorporation of one of the cold-inducible outer membrane proteins, OmpC176, was significantly increased in EMVs after the depletion of EPA. These results provide a basis for the

construction of an EMV-based, low-temperature protein production system and show the involvement of EPA in the regulation of EMV biogenesis.

3.2738 Macrophage-derived exosomes induce inflammatory factors in endothelial cells under hypertensive conditions

Osada-Oka, M., Shiota, M., Izumi, Y., Nishiyama, M., Tanaka, M., Yamaguchi, T., Sakurai, E., Miura, K. and Iwao, H.
Hypertension Res., ***40(4)***, *353-360 (2017)*

Hypertension is one of the most important cardiovascular risk factors and results in macrophage infiltration of blood vessels. However, how macrophages coordinate inflammatory responses with endothelial cells (ECs) remains unclear. In this study, we investigated whether exosomes upregulate the expression of inflammatory factors in ECs under hypertensive conditions. Hypertension was induced in rats by continuous infusion of angiotensin II (Ang II). Exosomes were purified from rat serum by density gradient and ultracentrifugation and used to stimulate human coronary artery ECs (HCAECs). Moreover, the interactions between HCAECs and exosomes from human THP-1-derived macrophages were analyzed. Administration of Ang II enhanced the expression of CD68, a macrophage marker, in rat hearts, suggesting enhanced infiltration of macrophages. In addition, the expression of intracellular adhesion molecule-1 (ICAM1) and plasminogen activator inhibitor-1 (PAI-1), a proinflammatory factor, was increased in hypertensive rat hearts compared with control rats. CD68 protein expression and an increase in the expression of some exosome markers were detected in exosomes from hypertensive rat serum. Moreover, the exosomes upregulated the expression levels of ICAM1 and PAI-1 in HCAECs. The level of miR-17, a negative regulator of ICAM1 expression, was markedly decreased in exosomes from hypertensive rat serum compared with exosomes from control rats. Interestingly, Ang II-stimulated THP-1-derived exosomes also enhanced the expression of ICAM1 and PAI-1 and contained reduced levels of miR-17 compared with exosomes from unstimulated cells. These results suggest that inflammation of ECs under hypertensive conditions is caused, at least in part, by macrophage-derived exosomes.

3.2739 The emerging role of exosome and microvesicle- (EMV-) based cancer therapeutics and immunotherapy

Moore, C., Kosgodage, U., lange, S. and Inal, J.M.
Int. J. Cancer, ***141(3)***, *428-436 (2017)*

There is an urgent need to develop new combination therapies beyond existing surgery, radio- and chemo-therapy, perhaps initially combining chemotherapy with the targeting specificities of immunotherapy. For this, strategies to limit inflammation and immunosuppression and evasion in the tumour microenvironment are also needed. To devise effective new immunotherapies we must first understand tumour immunology, including the roles of T cells, macrophages, myeloid suppressor cells and of exosomes and microvesicles (EMVs) in promoting angiogenesis, tumour growth, drug resistance and metastasis. One promising cancer immunotherapy discussed uses cationic liposomes carrying tumour RNA (RNA-lipoplexes) to provoke a strong anti-viral-like (cytotoxic $CD8^+$) anti-tumour immune response. Mesenchymal stem cell-derived EMVs, with their capacity to migrate towards inflammatory areas including solid tumours, have also been used. As tumour EMVs clearly exacerbate the tumour microenvironment, another therapy option could involve EMV removal. Affinity-based methods to deplete EMVs, including an immunodepletion, antibody-based affinity substrate, are therefore considered. Finally EMV and exosome-mimetic nanovesicles (NVs) delivery of siRNA or chemotherapeutic drugs that target tumours using peptide ligands for cognate receptors on the tumour cells are discussed. We also touch upon the reversal of drug efflux in EMVs from cancer cells which can sensitize cells to chemotherapy. The use of immunotherapy in combination with the advent of EMVs provides potent therapies to various cancers.

3.2740 Effect of circulating exosomes from transition cows on Madin-Darby bovine kidney cell function

Crookenden, M.A. et al
J. Dairy Sci., ***100(7)***, *5687-5700 (2017)*

The greatest risk of metabolic and infectious disease in dairy cows is during the transition from pregnancy to lactating (i.e., the transition period). The objective of this experiment was to determine the effects of extracellular vesicles (microvesicles involved in cell-to-cell signaling) isolated from transition cows on target cell function. We previously identified differences in the protein profiles of exosomes isolated from cows divergent in metabolic health status. Therefore, we hypothesized that these exosomes would affect target tissues differently. To investigate this, 2 groups of cows (n = 5/group) were selected based on the

concentration of β-hydroxybutyrate and fatty acids in plasma and triacylglycerol concentration in liver at wk 1 and 2 postcalving. Cows with high concentrations of β-hydroxybutyrate, fatty acids, and triacylglycerol were considered at increased risk of clinical disease during the transition period (high-risk group; n = 5) and were compared with cows that had low concentrations of the selected health indicators (low-risk group; n = 5). At 2 time points during the transition period (postcalving at wk 1 and 4), blood was sampled and plasma exosomes were isolated from the high-risk and low-risk cows. The exosomes were applied at concentrations of 10 and 1 μg/mL to 5×10^3 Madin-Darby bovine kidney cells grown to 50% confluence in 96-well plates. Results indicate a numerical increase in cell proliferation when exosomes from high-risk cows were applied compared with those from low-risk cows. Consistent with an effect on cell proliferation, quantitative reverse transcriptase PCR indicated a trend for upregulation of 3 proinflammatory genes (granulocyte colony-stimulating factor, ciliary neurotrophic factor, and CD27 ligand) with the application of high-risk exosomes, which are involved in cellular growth and survival. Proteomic analysis indicated 2 proteins in the low-risk group that were not identified in the high-risk group (endoplasmin and catalase), which may also be indicative of the metabolic state of origin. It is likely that the metabolic state of the transition cow affects cellular function through exosomal messaging; however, more in-depth research into cross-talk between exosomes and target cells is required to determine whether exosomes influence Madin-Darby bovine kidney cells in this manner.

3.2741 The role of lipid raft translocation of prohibitin in regulation of Akt and Raf-protected apoptosis of HaCaT cells upon ultraviolet B irradiation
Wu, Q. and Wu, S.
Mol. Carcinogenesis, ***56(7)***, *1789-1797 (2017)*

Prohibitin (PHB) plays a role in regulation of ultraviolet B light (UVB)-induced apoptosis of human keratinocytes, HaCaT cells. The regulatory function of PHB appears to be associated with its lipid raft translocation. However, the detailed mechanism for PHB-mediated apoptosis of these keratinocytes upon UVB irradiation is not clear. In this report, we determined the role of lipid raft trans location of PHB in regulation of UVB-induced apoptosis. Our data show that upon UVB irradiation PHB is translocated from the non-raft membrane to the lipid rafts, which is correlated with a release of both Akt and Raf from membrane. Overexpression of Akt and/or Raf impedes UVB-induced lipid raft translocation of PHB. Immunoprecipitation analysis indicates that UVB alters the interactions among PHB, Akt, and Raf. Reduced expression of PHB leads to a decreased phosphorylation of Akt and ERK, as well as a decreased activity of Akt, and increased apoptosis of the cells upon UVB irradiation. These results suggest that PHB regulates UVB-induced apoptosis of keratinocytes via a mechanism that involves detachment from Akt and Raf on the plasma membrane, and sequential lipid raft translocation.

3.2742 NCAM-140 Translocation into Lipid Rafts Mediates the Neuroprotective Effects of GDNF
Li, L., Chen, H., Wang, M., Chen, F., Gao, J., Sun, S., Li, Y. and Gao, D.
Mol. Neurobiol., ***54(4)***, *2739-2751 (2017)*

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor for substantia nigra dopaminergic (DA) neuronal cells. Recent studies have demonstrated that neural cell adhesion molecule functions as a signal transduction receptor for GDNF. The purpose of this study is to reveal whether neural cell adhesion molecule (NCAM) mediates the protective effects of GDNF on DA neuronal cells and further explore the mechanisms involved. We utilized SH-SY5Y cell line to establish a model of 6-hydroxydopamine (6-OHDA)-injured DA neuronal cells. Lentiviral vectors were constructed to knockdown or overexpress NCAM-140, and a density gradient centrifugation method was employed to separate membrane lipid rafts. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), flow cytometric analysis, and western blotting were used to evaluate the protective effects of GDNF. The results showed that GDNF could protect 6-OHDA-injured SH-SY5Y cells via improving cell viability and decreasing the cell death rate and cleaved caspase-3 expression. NCAM-140 knockdown decreased cell viability and increased the cell death rate and cleaved caspase-3 expression, while its overexpression had the opposite effects. Notably, the amount of NCAM-140 located in lipid rafts increased after GDNF treatment. Pretreatment with 2-bromopalmitate, a specific inhibitor of protein palmitoylation, suppressed NCAM-140 translocation to lipid rafts and reduced the NCAM-mediated protective effects of GDNF on injured DA neuronal cells. Our results suggest that GDNF have the protective effects on injured DA cells by influencing NCAM-140 translocation into lipid rafts.

3.2743 **Using hyperLOPIT to perform high-resolution mapping of the spatial proteome**
Mulvey, C.M., Breckels, L.M., Geladaki, A., Britovsek, N.K., Nightingale, D.J.H., Christoforou, A., Elzek, M., Deery, M.J., Gatto, L. and Lilley, K.S.
*Nature Protocols, **12(6)**, 1110-1135 (2017)*

The organization of eukaryotic cells into distinct subcompartments is vital for all functional processes, and aberrant protein localization is a hallmark of many diseases. Microscopy methods, although powerful, are usually low-throughput and dependent on the availability of fluorescent fusion proteins or highly specific and sensitive antibodies. One method that provides a global picture of the cell is localization of organelle proteins by isotope tagging (LOPIT), which combines biochemical cell fractionation using density gradient ultracentrifugation with multiplexed quantitative proteomics mass spectrometry, allowing simultaneous determination of the steady-state distribution of hundreds of proteins within organelles. Proteins are assigned to organelles based on the similarity of their gradient distribution to those of well-annotated organelle marker proteins. We have substantially re-developed our original LOPIT protocol (published by *Nature Protocols* in 2006) to enable the subcellular localization of thousands of proteins per experiment (hyperLOPIT), including spatial resolution at the suborganelle and large protein complex level. This Protocol Extension article integrates all elements of the hyperLOPIT pipeline, including an additional enrichment strategy for chromatin, extended multiplexing capacity of isobaric mass tags, state-of-the-art mass spectrometry methods and multivariate machine-learning approaches for analysis of spatial proteomics data. We have also created an open-source infrastructure to support analysis of quantitative mass-spectrometry-based spatial proteomics data (**http://bioconductor.org/packages/pRoloc**) and an accompanying interactive visualization framework (**http://www.bioconductor.org/packages/pRolocGUI**). The procedure we outline here is applicable to any cell culture system and requires ~1 week to complete sample preparation steps, ~2 d for mass spectrometry data acquisition and 1–2 d for data analysis and downstream informatics.

3.2744 **Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer**
Kamerkar, S., LebLeu, V.S., Sugimoto, H., Yang, S., Ruivo, C.F., Melo, S.A., Lee, J.J. and Kalhuri, R.
*Nature, **546**, 498-503 (2017)*

The mutant form of the GTPase KRAS is a key driver of pancreatic cancer but remains a challenging therapeutic target. Exosomes are extracellular vesicles generated by all cells, and are naturally present in the blood. Here we show that enhanced retention of exosomes, compared to liposomes, in the circulation of mice is likely due to CD47-mediated protection of exosomes from phagocytosis by monocytes and macrophages. Exosomes derived from normal fibroblast-like mesenchymal cells were engineered to carry short interfering RNA or short hairpin RNA specific to oncogenic $Kras^{G12D}$, a common mutation in pancreatic cancer. Compared to liposomes, the engineered exosomes (known as iExosomes) target oncogenic KRAS with an enhanced efficacy that is dependent on CD47, and is facilitated by macropinocytosis. Treatment with iExosomes suppressed cancer in multiple mouse models of pancreatic cancer and significantly increased overall survival. Our results demonstrate an approach for direct and specific targeting of oncogenic KRAS in tumours using iExosomes.

3.2745 **The Glycosyltransferase QUA1 Regulates Chloroplast-Associated Calcium Signaling During Salt and Drought Stress in Arabidopsis**
Zheng, Y., Liao, C., Zhao, S., Wang, C. and Guo, Y.
*Plant Cell Physiol., **58(2)**, 329-341 (2017)*

Cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation induced by various signals is responsible for appropriate downstream responses. Through a genetic screen of *Arabidopsis thaliana* mutants defective in stress-induced $[Ca^{2+}]_{cyt}$ elevation, the glycosyltransferase QUASIMODO1 (QUA1) was identified as a regulator of $[Ca^{2+}]_{cyt}$ in response to salt stress. Compared with the wild type, the *qua1-4* mutant exhibited a dramatically greater increase in $[Ca^{2+}]_{cyt}$ under NaCl treatment. Functional analysis showed that QUA1 is a novel chloroplast protein that regulates cytoplasmic Ca^{2+} signaling. QUA1 was detected in chloroplast thylakoids, and the *qua1-4* mutant exhibited irregularly stacked grana. The observed greater increase in $[Ca^{2+}]_{cyt}$ was inhibited upon recovery of chloroplast function in the *qua1-4* mutant. Further analysis showed that CAS, a thylakoid-localized calcium sensor, also displayed irregularly stacked grana, and the chloroplasts of the *qua1-4 cas-1* double mutant were similar to those of *cas-1* plants. In QUA1-overexpressing plants, the protein level of CAS was decreased, and CAS was readily degraded under osmotic stress. When *CAS* was silenced in the *qua1-4* mutant, the large $[Ca^{2+}]_{cyt}$ increase was blocked, and the higher expression of *PLC3* and *PLC4* was suppressed. Under osmotic stress, the *qua1-4* mutant showed

an even greater elevation in $[Ca^{2+}]_{cyt}$ and was hypersensitive to drought stress. However, this sensitivity was inhibited when the increase in $[Ca^{2+}]_{cyt}$ was repressed in the *qua1-4* mutant. Collectively, our data indicate that QUA1 may function in chloroplast-dependent calcium signaling under salt and drought stresses. Additionally, CAS may function downstream of QUA1 to mediate these processes.

3.2746 A voltage-dependent K+ channel in the lysosome is required for refilling lysosomal Ca2+ stores
Wang, W., Zhang, X., Gao, Q., Lawas, M., Yu, L., Cheng, X., Gu, M., Sahoo, N., Li, X., Li, P., Ireland, S., Meredith, A. and Xu, H.
J. Cell Biol., ***216(6)***, *1715-1730 (2017)*

The resting membrane potential (Δψ) of the cell is negative on the cytosolic side and determined primarily by the plasma membrane's selective permeability to K^+. We show that lysosomal Δψ is set by lysosomal membrane permeabilities to Na^+ and H^+, but not K^+, and is positive on the cytosolic side. An increase in juxta-lysosomal Ca^{2+} rapidly reversed lysosomal Δψ by activating a large voltage-dependent and K^+-selective conductance ($LysoK_{VCa}$). $LysoK_{VCa}$ is encoded molecularly by SLO1 proteins known for forming plasma membrane BK channels. Opening of single $LysoK_{VCa}$ channels is sufficient to cause the rapid, striking changes in lysosomal Δψ. Lysosomal Ca^{2+} stores may be refilled from endoplasmic reticulum (ER) Ca^{2+} via ER–lysosome membrane contact sites. We propose that $LysoK_{VCa}$ serves as the perilysosomal Ca^{2+} effector to prime lysosomes for the refilling process. Consistently, genetic ablation or pharmacological inhibition of $LysoK_{VCa}$, or abolition of its Ca^{2+} sensitivity, blocks refilling and maintenance of lysosomal Ca^{2+} stores, resulting in lysosomal cholesterol accumulation and a lysosome storage phenotype.

3.2747 COPII-coated membranes function as transport carriers of intracellular procollagen I
Gorur, A., Yuan, L., Kenny, S.J., Baba, S., Xu, K. and Schekman, R.
J. Cell Biol., ***216(6)***, *1745-1759 (2017)*

The coat protein complex II (COPII) is essential for the transport of large cargo, such as 300-nm procollagen I (PC1) molecules, from the endoplasmic reticulum (ER) to the Golgi. Previous work has shown that the CUL3-KLHL12 complex increases the size of COPII vesicles at ER exit sites to more than 300 nm in diameter and accelerates the secretion of PC1. However, the role of large COPII vesicles as PC1 transport carriers was not unambiguously demonstrated. In this study, using stochastic optical reconstruction microscopy, correlated light electron microscopy, and live-cell imaging, we demonstrate the existence of mobile COPII-coated vesicles that completely encapsulate the cargo PC1 and are physically separated from ER. We also developed a cell-free COPII vesicle budding reaction that reconstitutes the capture of PC1 into large COPII vesicles. This process requires COPII proteins and the GTPase activity of the COPII subunit SAR1. We conclude that large COPII vesicles are bona fide carriers of PC1.

3.2748 Schistosomal MicroRNAs Isolated From Extracellular Vesicles in Sera of Infected Patients: A New Tool for Diagnosis and Follow-up of Human Schistosomiasis
Meningher, T., Lerman, G., Regev-Rudzki, N., Gold, D., Ben-Dov, I.Z., Sidi, Y., Avni, D. and Schwartz, E.
J. Infect. Dis., ***215(3)***, *378-386 (2017)*

Background.
Schistosomiasis traditionally has been diagnosed by detecting eggs in stool or urine. However, the sensitivity of these examinations is limited, especially in travelers with a low worm burden. Serologic tests have a greater sensitivity, but their results remain positive regardless of treatment and thus cannot be used for follow-up of patients. We hypothesized that detection of worm microRNAs (miRNAs) in serum can overcome the drawbacks of the existing diagnostic methods.
Methods and Results.
Twenty-six returning travelers with schistosomiasis (based on positive results of serologic tests or detection of ova) and 17 healthy controls were included in the study. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) amplification of miRNA extracted directly from 500 μL of serum had limited sensitivity and specificity. However, qRT-PCR analysis of RNA extracted from 200 μL of serum extracellular vesicles detected 4 schistosomal miRNAs; the sensitivity and specificity of the 2 highest expressed miRNAs (bantam and miR-2c-3p) were 86% and 84%, respectively. In 7 patients with posttreatment serum available for analysis, we observed outcomes ranging from a reduction in the schistosomal miRNA level to full recovery from disease.
Conclusions.

qRT-PCR of pathogen miRNAs isolated from extracellular vesicles in sera from infected individuals may provide a new tool for diagnosing schistosomiasis in patients with a low parasite burden. This assay could also be used for evaluating the outcome of therapy, as well as disease-control programs.

3.2749 Caveolins and cavins in the trafficking, maturation, and degradation of caveolae: implications for cell physiology

Busija, A.R., Patel, H.H. and Insel, P.A.
Am. J. Physiol. Cell Physiol., ***312***, *C459-C477 (2017)*

Caveolins (Cavs) are ~20 kDa scaffolding proteins that assemble as oligomeric complexes in lipid raft domains to form caveolae, flask-shaped plasma membrane (PM) invaginations. Caveolae ("little caves") require lipid-lipid, protein-lipid, and protein-protein interactions that can modulate the localization, conformational stability, ligand affinity, effector specificity, and other functions of proteins that are partners of Cavs. Cavs are assembled into small oligomers in the endoplasmic reticulum (ER), transported to the Golgi for assembly with cholesterol and other oligomers, and then exported to the PM as an intact coat complex. At the PM, cavins, ~50 kDa adapter proteins, oligomerize into an outer coat complex that remodels the membrane into caveolae. The structure of caveolae protects their contents (i.e., lipids and proteins) from degradation. Cellular changes, including signal transduction effects, can destabilize caveolae and produce cavin dissociation, restructuring of Cav oligomers, ubiquitination, internalization, and degradation. In this review, we provide a perspective of the life cycle (biogenesis, degradation), composition, and physiologic roles of Cavs and caveolae and identify unanswered questions regarding the roles of Cavs and cavins in caveolae and in regulating cell physiology.[1]

3.2750 β-Adrenergic induction of lipolysis in hepatocytes is inhibited by ethanol exposure

Schott, M.B., Rasineni, K., Weller, S.W., Schulze, R.J., Sletten, A.C., Casey, C.A. and McNiven, M.A.
J. Biol. Chem., ***292(28)***, *11815-11828 (2017)*

In liver steatosis (*i.e.* fatty liver), hepatocytes accumulate many large neutral lipid storage organelles known as lipid droplets (LDs). LDs are important in the maintenance of energy homeostasis, but the signaling mechanisms that stimulate LD metabolism in hepatocytes are poorly defined. In adipocytes, catecholamines target the β-adrenergic (β-AR)/cAMP pathway to activate cytosolic lipases and induce their recruitment to the LD surface. Therefore, the goal of this study was to determine whether hepatocytes, like adipocytes, also undergo cAMP-mediated lipolysis in response to β-AR stimulation. Using primary rat hepatocytes and human hepatoma cells, we found that treatment with the β-AR agent isoproterenol caused substantial LD loss via activation of cytosolic lipases adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). β-Adrenergic stimulation rapidly activated PKA, which led to the phosphorylation of ATGL and HSL and their recruitment to the LD surface. To test whether this β-AR-dependent lipolysis pathway was altered in a model of alcoholic fatty liver, primary hepatocytes from rats fed a 6-week EtOH-containing Lieber-DeCarli diet were treated with cAMP agonists. Compared with controls, EtOH-exposed hepatocytes showed a drastic inhibition in β-AR/cAMP-induced LD breakdown and the phosphorylation of PKA substrates, including HSL. This observation was supported in VA-13 cells, an EtOH-metabolizing human hepatoma cell line, which displayed marked defects in both PKA activation and isoproterenol-induced ATGL translocation to the LD periphery. In summary, these findings suggest that β-AR stimulation mobilizes cytosolic lipases for LD breakdown in hepatocytes, and perturbation of this pathway could be a major consequence of chronic EtOH insult leading to fatty liver.

3.2751 Hepatitis C Virus Lipoviroparticles Assemble in the Endoplasmic Reticulum (ER) and Bud off from the ER to the Golgi Compartment in COPII Vesicles

Syed, G.H., Khan, M., Yang, S. and Siddiqui, A.
J. Virol., ***91(15)***, *e00499-17 (2017)*

Hepatitis C virus (HCV) exists as a lipoprotein-virus hybrid lipoviroparticle (LVP). *In vitro* studies have demonstrated the importance of apolipoproteins in HCV secretion and infectivity, leading to the notion that HCV coopts the secretion of very-low-density lipoprotein (VLDL) for its egress. However, the mechanisms involved in virus particle assembly and egress are still elusive. The biogenesis of VLDL particles occurs in the endoplasmic reticulum (ER), followed by subsequent lipidation in the ER and Golgi compartment. The secretion of mature VLDL particles occurs through the Golgi secretory pathway. HCV virions are believed to latch onto or fuse with the nascent VLDL particle in either the ER or the Golgi compartment, resulting in the generation of LVPs. In our attempt to unravel the collaboration between HCV and VLDL secretion, we studied HCV particles budding from the ER *en route* to the Golgi

compartment in COPII vesicles. Biophysical characterization of COPII vesicles fractionated on an iodixanol gradient revealed that HCV RNA is enriched in the highly buoyant COPII vesicle fractions and cofractionates with apolipoprotein B (ApoB), ApoE, and the HCV core and envelope proteins. Electron microscopy of immunogold-labeled microsections revealed that the HCV envelope and core proteins colocalize with apolipoproteins and HCV RNA in Sec31-coated COPII vesicles. Ultrastructural analysis also revealed the presence of HCV structural proteins, RNA, and apolipoproteins in the Golgi stacks. These findings support the hypothesis that HCV LVPs assemble in the ER and are transported to the Golgi compartment in COPII vesicles to embark on the Golgi secretory route.

3.2752 Exosomes from metastatic cancer cells transfer amoeboid phenotype to non-metastatic cells and increase endothelial permeability: their emerging role in tumor heterogeneity
Schillaci, O., Fontana, s., Monteleone, F., Taverna, S., Di Bella, M.A., Di Vizio, D. And Alessandro, R.
Scientific Reports, 7:4711 (2017)

The goal of this study was to understand if exosomes derived from high-metastatic cells may influence the behavior of less aggressive cancer cells and the properties of the endothelium. We found that metastatic colon cancer cells are able to transfer their amoeboid phenotype to isogenic primary cancer cells through exosomes, and that this morphological transition is associated with the acquisition of a more aggressive behavior. Moreover, exosomes from the metastatic line (SW620Exos) exhibited higher ability to cause endothelial hyperpermeability than exosomes from the non metastatic line (SW480Exos). SWATH-based quantitative proteomic analysis highlighted that SW620Exos are significantly enriched in cytoskeletal-associated proteins including proteins activating the RhoA/ROCK pathway, known to induce amoeboid properties and destabilization of endothelial junctions. In particular, thrombin was identified as a key mediator of the effects induced by SW620Exos in target cells, in which we also found a significant increase of RhoA activity. Overall, our results demonstrate that in a heterogeneous context exosomes released by aggressive sub-clones can contribute to accelerate tumor progression by spreading malignant properties that affect both the tumor cell plasticity and the endothelial cell behavior.

3.2753 Directional Exosome Proteomes Reflect Polarity-Specific Functions in Retinal Pigmented Epithelium Monolayers
Klingeborn, M., Dismuke, W.M., Skiba, N.P., Kelly, U., Stamer, W.D. and Bowes Rickman, C.
Scientific Reports, 7:4901 (2017)

The retinal pigmented epithelium (RPE) forms the outer blood-retinal barrier in the eye and its polarity is responsible for directional secretion and uptake of proteins, lipoprotein particles and extracellular vesicles (EVs). Such a secretional division dictates directed interactions between the systemic circulation (basolateral) and the retina (apical). Our goal is to define the polarized proteomes and physical characteristics of EVs released from the RPE. Primary cultures of porcine RPE cells were differentiated into polarized RPE monolayers on permeable supports. EVs were isolated from media bathing either apical or basolateral RPE surfaces, and two subpopulations of small EVs including exosomes, and dense EVs, were purified and processed for proteomic profiling. In parallel, EV size distribution and concentration were determined. Using protein correlation profiling mass spectrometry, a total of 631 proteins were identified in exosome preparations, 299 of which were uniquely released apically, and 94 uniquely released basolaterally. Selected proteins were validated by Western blot. The proteomes of these exosome and dense EVs preparations suggest that epithelial polarity impacts directional release. These data serve as a foundation for comparative studies aimed at elucidating the role of exosomes in the molecular pathophysiology of retinal diseases and help identify potential therapeutic targets and biomarkers.

3.2754 DGKδ triggers endoplasmic reticulum release of IFT88-containing vesicles destined for the assembly of primary cilia
Ding, J., Shao, L., Yao, Y., Tong, X., Liu, H., Yue, S., Xie, L. and Cheng, S.Y.
Scientific Reports, 7:5296 (2017)

The morphogenic factor Sonic hedgehog (Shh) signals through the primary cilium, which relies on intraflagellar transport to maintain its structural integrity and function. However, the process by which protein and lipid cargos are delivered to the primary cilium from their sites of synthesis still remains poorly characterized. Here, we report that diacylglycerol kinase δ (DGKδ), a residential lipid kinase in the endoplasmic reticulum, triggers the release of IFT88-containing vesicles from the ER exit sites (ERES), thereby setting forth their movement to the primary cilium. Encoded by the gene whose mutations originally implicated the primary cilium as the venue of Shh signaling, IFT88 is known to be part of the

complex B that drives the anterograde transport within cilia. We show that IFT88 interacts with DGKδ, and is associated with COPII-coated vesicles at the ERES. Using a combination of RNAi silencing and gene knockout strategies, we further show that DGKδ is required for supporting Shh signaling both *in vitro* and *in vivo*, demonstrating the physiological significance of this regulation.

3.2755 Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth

Samuel, M., Chisanga, D., Liern, M., Keerthikumar, S., Anand, S., Ang, C-S., Adda, C.G., Versteegen, E., Jois, M. and Mathivanan, S.
Scientific Reports, 7:5933 (2017)

Exosomes are extracellular vesicles secreted by multiple cell types into the extracellular space. They contain cell-state specific cargos which often reflects the (patho)physiological condition of the cells/organism. Milk contains high amounts of exosomes and it is unclear whether their cargo is altered based on the lactation stage of the organism. Here, we isolated exosomes from bovine milk that were obtained at various stages of lactation and examined the content by quantitative proteomics. Exosomes were isolated by OptiPrep density gradient centrifugation from milk obtained from cow after 24, 48 and 72 h post calving. As control, exosomes were also isolated from cows during mid-lactation period which has been referred to as mature milk (MM). Biochemical and biophysical characterization of exosomes revealed the high abundance of exosomes in colostrum and MM samples. Quantitative proteomics analysis highlighted the change in the proteomic cargo of exosomes based on the lactation state of the cow. Functional enrichment analysis revealed that exosomes from colostrum are significantly enriched with proteins that can potentially regulate the immune response and growth. This study highlights the importance of exosomes in colostrum and hence opens up new avenues to exploit these vesicles in the regulation of the immune response and growth.

3.2756 Loss of Calreticulin Uncovers a Critical Role for Calcium in Regulating Cellular Lipid Homeostasis

Wang, W-A., Liu, W-X., Durnaoglu, S., Lee, S-K., Lian, J., Lehner, R., Ahnn, J., Agellon, L.B. and Michalak, M.
Scientific Reports, 7:5941 (2017)

A direct link between Ca^{2+} and lipid homeostasis has not been definitively demonstrated. In this study, we show that manipulation of ER Ca^{2+} causes the re-distribution of a portion of the intracellular unesterified cholesterol to a pool that is not available to the SCAP-SREBP complex. The SREBP processing pathway in ER Ca^{2+} depleted cells remained fully functional and responsive to changes in cellular cholesterol status but differed unexpectedly in basal activity. These findings establish the role of Ca^{2+} in determining the reference set-point for controlling cellular lipid homeostasis. We propose that ER Ca^{2+} status is an important determinant of the basal sensitivity of the sterol sensing mechanism inherent to the SREBP processing pathway.

3.2757 Sphingomimetic multiple sclerosis drug FTY720 activates vesicular synaptobrevin and augments neuroendocrine secretion

Darios, F.D., Jorgacevski, J., Flasker, A., Zorec, R., Garcia-Martinez, V., Villanueva, J., Gutierrez, L.M., Leese, C., Bal, M., Nosyreva, E., Kavalali, E.T. and Davletov, B.
Scientific Reports, 7:5958 (2017)

Neurotransmission and secretion of hormones involve a sequence of protein/lipid interactions with lipid turnover impacting on vesicle trafficking and ultimately fusion of secretory vesicles with the plasma membrane. We previously demonstrated that sphingosine, a sphingolipid metabolite, promotes formation of the SNARE complex required for membrane fusion and also increases the rate of exocytosis in isolated nerve terminals, neuromuscular junctions, neuroendocrine cells and in hippocampal neurons. Recently a fungi-derived sphingosine homologue, FTY720, has been approved for treatment of multiple sclerosis. In its non-phosphorylated form FTY720 accumulates in the central nervous system, reaching high levels which could affect neuronal function. Considering close structural similarity of sphingosine and FTY720 we investigated whether FTY720 has an effect on regulated exocytosis. Our data demonstrate that FTY720 can activate vesicular synaptobrevin for SNARE complex formation and enhance exocytosis in neuroendocrine cells and neurons.

3.2758 Primary fibroblasts from CSPα mutation carriers recapitulate hallmarks of the adult onset neuronal ceroid lipofuscinosis
Benitez, B. and Sands, M.S.
*Scientific Reports, **7**:6332 (2017)*

Mutations in the co- chaperone protein, CSPα, cause an autosomal dominant, adult-neuronal ceroid lipofuscinosis (AD-ANCL). The current understanding of CSPα function exclusively at the synapse fails to explain the autophagy-lysosome pathway (ALP) dysfunction in cells from AD-ANCL patients. Here, we demonstrate unexpectedly that primary dermal fibroblasts from pre-symptomatic mutation carriers recapitulate *in vitro* features found in the brains of AD-ANCL patients including auto-fluorescent storage material (AFSM) accumulation, CSPα aggregates, increased levels of lysosomal proteins and lysosome enzyme activities. AFSM accumulation correlates with CSPα aggregation and both are susceptible to pharmacological modulation of ALP function. In addition, we demonstrate that endogenous CSPα is present in the lysosome-enriched fractions and co-localizes with lysosome markers in soma, neurites and synaptic boutons. Overexpression of CSPα wild-type (WT) decreases lysotracker signal, secreted lysosomal enzymes and SNAP23-mediated lysosome exocytosis. CSPα WT, mutant and aggregated CSPα are degraded mainly by the ALP but this disease-causing mutation exhibits a faster rate of degradation. Co-expression of both WT and mutant CSPα cause a block in the fusion of autophagosomes/lysosomes. Our data suggest that aggregation-dependent perturbation of ALP function is a relevant pathogenic mechanism for AD-ANCL and supports the use of AFSM or CSPα aggregation as biomarkers for drug screening purposes.

3.2759 Outer membrane vesicle-associated lipase FtlA enhances cellular invasion and virulence in Francisella tularensis LVS
Chen, F., Cui, G., Wang, S., Nair, M.K.M., He, L., Qi, X., Han, X., Zhang, H., Zhang, J-R. and Su, J.
*Emerging Microbes & Infections, **6**, e66 (2017)*

Francisella tularensis is a highly infectious intracellular pathogen that infects a wide range of host species and causes fatal pneumonic tularemia in humans. *ftlA* was identified as a potential virulence determinant of the *F. tularensis* live vaccine strain (LVS) in our previous transposon screen, but its function remained undefined. Here, we show that an unmarked deletion mutant of *ftlA* was avirulent in a pneumonia mouse model with a severely impaired capacity to infect host cells. Consistent with its sequence homology with GDSL lipase/esterase family proteins, the FtlA protein displayed lipolytic activity in both *E. coli* and *F. tularensis* with a preference for relatively short carbon-chain substrates. FtlA thus represents the first *F. tularensis* lipase to promote bacterial infection of host cells and *in vivo* fitness. As a cytoplasmic protein, we found that FtlA was secreted into the extracellular environment as a component of outer membrane vesicles (OMVs). Further confocal microscopy analysis revealed that the FtlA-containing OMVs isolated from *F. tularensis* LVS attached to the host cell membrane. Finally, the OMV-associated FtlA protein complemented the genetic deficiency of the Δ*ftlA* mutant in terms of host cell infection when OMVs purified from the parent strain were co-incubated with the mutant bacteria. These lines of evidence strongly suggest that the FtlA lipase promotes *F. tularensis* adhesion and internalization by modifying bacterial and/or host molecule(s) when it is secreted as a component of OMVs.

3.2760 Extracellular vesicles from human liver stem cells restore argininosuccinate synthase deficiency
Sanchez, M.B.H., Previdi, S., Bruno, S., Fonsato, V., Deregibus, M.C., Kholia, S., petrillo, S., Tolosano, E., Critelli, R., Spada, M., Romagnoli, R., Salizzoni, M., Tetta, C. and Camussi, G.
*Stem Cell Res. Ther., **8**:176 (2017)*

Argininosuccinate synthase (ASS)1 is a urea cycle enzyme that catalyzes the conversion of citrulline and aspartate to argininosuccinate. Mutations in the ASS1 gene cause citrullinemia type I, a rare autosomal recessive disorder characterized by neonatal hyperammonemia, elevated citrulline levels, and early neonatal death. Treatment for this disease is currently restricted to liver transplantation; however, due to limited organ availability, substitute therapies are required. Recently, extracellular vesicles (EVs) have been reported to act as intercellular transporters carrying genetic information responsible for cell reprogramming. In previous studies, we isolated a population of stem cell-like cells known as human liver stem cells (HLSCs) from healthy liver tissue. Moreover, EVs derived from HLSCs were reported to exhibit regenerative effects on the liver parenchyma in models of acute liver injury. The aim of this study was to evaluate whether EVs derived from normal HLSCs restored ASS1 enzymatic activity and urea production in hepatocytes differentiated from HLSCs derived from a patient with type I citrullinemia.

3.2761 Saccharomyces cerevisiae cells lacking Pex3 contain membrane vesicles that harbor a subset of peroxisomal membrane proteins

Wroblewska, J.P., Cruz-Zaragoza, L.D., Yan, W., Schummer, A., Chuartzman, S.G., de Boer, R., Oeljekaus, S., Schuldiner, M., Zalckvar, E., Warscheid, B., Erdmann, R. and van der Klei, I.J.
*BBA – Mol Cell Res., **1864**, 1656-1667 (2017)*

Pex3 has been proposed to be important for the exit of peroxisomal membrane proteins (PMPs) from the ER, based on the observation that PMPs accumulate at the ER in *Saccharomyces cerevisiae pex3* mutant cells.
Using a combination of microscopy and biochemical approaches, we show that a subset of the PMPs, including the receptor docking protein Pex14, localizes to membrane vesicles in *S. cerevisiae pex3* cells. These vesicles are morphologically distinct from the ER and do not co-sediment with ER markers in cell fractionation experiments. At the vesicles, Pex14 assembles with other peroxins (Pex13, Pex17, and Pex5) to form a complex with a composition similar to the PTS1 import pore in wild-type cells.
Fluorescence microscopy studies revealed that also the PTS2 receptor Pex7, the importomer organizing peroxin Pex8, the ubiquitin conjugating enzyme Pex4 with its recruiting PMP Pex22, as well as Pex15 and Pex25 co-localize with Pex14. Other peroxins (including the RING finger complex and Pex27) did not accumulate at these structures, of which Pex11 localized to mitochondria. In line with these observations, proteomic analysis showed that in addition to the docking proteins and Pex5, also Pex7, Pex4/Pex22 and Pex25 were present in Pex14 complexes isolated from *pex3* cells. However, formation of the entire importomer was not observed, most likely because Pex8 and the RING proteins were absent in the Pex14 protein complexes.
Our data suggest that peroxisomal membrane vesicles can form in the absence of Pex3 and that several PMPs can insert in these vesicles in a Pex3 independent manner.

3.2762 Adaptations in rod outer segment disc membranes in response to environmental lighting conditions

Rakshit, T., Senapati, S., Parmar, V.M., Sahu, B., Maeda, A. and Park, P.S-H.
*BBA – Mol. Cell Res., **1864**, 1691-1702 (2017)*

The light-sensing rod photoreceptor cell exhibits several adaptations in response to the lighting environment. While adaptations to short-term changes in lighting conditions have been examined in depth, adaptations to long-term changes in lighting conditions are less understood. Atomic force microscopy was used to characterize the structure of rod outer segment disc membranes, the site of photon absorption by the pigment rhodopsin, to better understand how photoreceptor cells respond to long-term lighting changes. Structural properties of the disc membrane changed in response to housing mice in constant dark or light conditions and these adaptive changes required output from the phototransduction cascade initiated by rhodopsin. Among these were changes in the packing density of rhodopsin in the membrane, which was independent of rhodopsin synthesis and specifically affected scotopic visual function as assessed by electroretinography. Studies here support the concept of photostasis, which maintains optimal photoreceptor cell function with implications in retinal degenerations.

3.2763 Recent advances on extracellular vesicles in therapeutic delivery: Challenges, solutions, and opportunities

Lu, M., Xing, H., Yang, Z., Sun, Y., Yang, T., Zhao, X., Cai, C., Wang, D. and Ding, P.
*Eur. J. Pharmaceut. Biopharmaceut., **119**, 381-395 (2017)*

Extracellular vesicles (EVs) are intrinsic mediators of intercellular communication in our body, allowing functional transfer of biomolecules (lipids, proteins, and nucleic acid) between diverse locations. Such an instrumental role evokes a surge of interest within the drug delivery community in tailoring EVs for therapeutic delivery. These vesicles represent a novel generation of drug delivery systems, providing high delivery efficiency, intrinsic targeting properties, and low immunogenicity. In the recent years, considerable research efforts have been directed toward developing safe and efficient EV-based delivery vehicles. Although EVs are shown to harbor great promise in therapeutic delivery, substantial improvements in exploring standardized isolation techniques with high efficiency and robust yield, scalable production, standard procedures for EV storage, efficient loading methods without damaging EV integrity, understanding their *in vivo* trafficking, and developing novel EV-based nanocarriers are still required before their clinical transformation. In this review, we seek to summarize the recent advance on harnessing EVs for drug delivery with focus on state-of-the-art solutions for overcoming major challenges.

3.2764 **Phytol-induced pathology in 2-hydroxyacyl-CoA lyase (HACL1) deficient mice. Evidence for a second non-HACL1-related lyase**
Mezzar, S., De Schryver, E., Asselberghs, S., Meyhi, E., Morvay, P.L., Baes, M. and Van Veldhoven, P.P.
*BBA – Mol. Cell Biol. Lipids, **1862**, 972-990 (2017)*

2-Hydroxyacyl-CoA lyase (HACL1) is a key enzyme of the peroxisomal α-oxidation of phytanic acid. To better understand its role in health and disease, a mouse model lacking HACL1 was investigated. Under normal conditions, these mice did not display a particular phenotype. However, upon dietary administration of phytol, phytanic acid accumulated in tissues, mainly in liver and serum of KO mice. As a consequence of phytanic acid (or a metabolite) toxicity, KO mice displayed a significant weight loss, absence of abdominal white adipose tissue, enlarged and mottled liver and reduced hepatic glycogen and triglycerides. In addition, hepatic PPARα was activated. The central nervous system of the phytol-treated mice was apparently not affected. In addition, 2OH-FA did not accumulate in the central nervous system of HACL1 deficient mice, likely due to the presence in the endoplasmic reticulum of an alternate HACL1-unrelated lyase. The latter may serve as a backup system in certain tissues and account for the formation of pristanic acid in the phytol-fed KO mice. As the degradation of pristanic acid is also impaired, both phytanoyl- and pristanoyl-CoA levels are increased in liver, and the ω-oxidized metabolites are excreted in urine. In conclusion, HACL1 deficiency is not associated with a severe phenotype, but in combination with phytanic acid intake, the normal situation in man, it might present with phytanic acid elevation and resemble a Refsum like disorder.

3.2765 **Roles of exosomes in the normal and diseased eye**
Klingeborn, M., Dismuke, W.M., Bowes Rickman, C.and Stamer, W.D.
*Progress in Retinal and Eye Res., **59**, 158-177 (2017)*

Exosomes are nanometer-sized vesicles that are released by cells in a controlled fashion and mediate a plethora of extra- and intercellular activities. Some key functions of exosomes include cell-cell communication, immune modulation, extracellular matrix turnover, stem cell division/differentiation, neovascularization and cellular waste removal. While much is known about their role in cancer, exosome function in the many specialized tissues of the eye is just beginning to undergo rigorous study. Here we review current knowledge of exosome function in the visual system in the context of larger bodies of data from other fields, in both health and disease. Additionally, we discuss recent advances in the exosome field including use of exosomes as a therapeutic vehicle, exosomes as a source of biomarkers for disease, plus current standards for isolation and validation of exosome populations. Finally, we use this foundational information about exosomes in the eye as a platform to identify areas of opportunity for future research studies.

3.2766 **Extracellular vesicles**
Simpson, R.J.
*Seminars in Cell and Develop. Biol., **67**, 1-2 (2017)*

Over the past decade extracellular vesicles (EVs) have been the subject of intense interest by investigators from diverse fields of biology. This has been largely due to their role in intercellular communication and the presence of diverse cargo such as proteins (including oncoproteins, tumor suppressors, transcriptional regulators, splicing factors etc), RNAs (microRNA, mRNA, lncRNA etc), DNA, and lipids [1]. It is now recognized that the function of EVs is dependent on the cargo they carry, which upon uptake by recipient cells can induce profound phenotypic changes. This horizontal transfer of bioactive molecules plays a vital role in tumor invasion and metastasis, immune modulation within the tumor microenvironment (TME), inflammation, epithelial-mesenchymal transition, neurobiology, pathogen dissemination – to name a few. It is now widely recognized that there are two major classes of EVs – shed microvesicles (sMVs), that are also referred to as microparticles, and exosomes [2]. sMVs are formed by the outward budding and abscission of the plasma membrane, whereas exosomes originate from preformed multivesicular bodies that traffic to the plasma membrane whereupon fusion with the plasma membrane they release their contents (exosomes) into the extracellular environment [1]. sMVs and exosomes exhibit different protein and RNA profiles and biophysical properties. While sMVs are heterogeneous in size (100 nm to > 1300 nm), exosomes are essentially homogeneous with a mean diameter ~36 nm [3]; the buoyant densities of sMVs (1.1–1.2 g/mL) and exosomes (1.1 g/mL), assessed by iodixanol density gradient centrifugation, tend to overlap. However, an interesting development in the EV field over the past 5 years has been the recognition that exosomes and sMVs can be further fractionated into discrete sub-populations, based upon cell polarity [4,5], buoyant density [6,7] and mechanism of biogenesis [8], that display

different bioactive molecule compositions. At this juncture it has been difficult to discern functional differences between EV subtypes.

3.2767 Leishmania donovani restricts mitochondrial dynamics to enhance miRNP stability and target RNA repression in host macrophages
Chakrabarty, Y. and Bhattacharyya, S.N.
*Mol. Biol. Cell, **28**, 2091-2105 (2017)*

MicroRNAs (miRNAs), the tiny regulatory RNAs, form complexes with Argonaute (Ago) proteins and inhibit gene expression in metazoan cells. While studying parasite-invaded macrophages, we identify a unique mode of gene regulation in which the parasite *Leishmania donovani* (*Ld*) causes mitochondrial depolarization, reduces mitochondrial dynamics, and restricts turnover of cellular microRNA ribonucleoprotein (miRNP) complexes in infected host cells. This leads to increased stability of miRNPs along with elevated levels of Ago2-bound cytokine mRNA in *Ld*-infected macrophages. Thus the increase of miRNP stability in *Ld*-infected cells curtails production of proinflammatory cytokines, which are otherwise detrimental for survival of the parasite within the infected macrophages. Loss of mitochondrial membrane potential is accompanied by reduced juxtaposition of endoplasmic reticulum (ER) and mitochondria as well as endosomes. This is likely coupled with enhanced sequestration and stabilization of ER- associated miRNPs observed in infected macrophage cells. Mitofusin 2 (Mfn2), a membrane protein implicated in ER–mitochondria tethering, also shows reduced expression in *Ld*-infected cells. A mitochondrial role in *Ld*-induced alteration of miRNA activity and stability is further corroborated by impaired compartmentalization and stabilization of miRNP components in Mfn2-depleted mammalian cells.

3.2768 Drosophila TG-A transglutaminase is secreted via an unconventional Golgi-independent mechanism involving exosomes and two types of fatty acylations
Shibata, T., Hadano, J., Kawasaki, D., Dong, X. and Kawabata, S-i.
*J. Biol. Chem., **292(25)**, 10723-10734 (2017)*

Transglutaminases (TGs) play essential intracellular and extracellular roles by covalently cross-linking many proteins. *Drosophila* TG is encoded by one gene and has two alternative splicing-derived isoforms, TG-A and TG-B, which contain distinct N-terminal 46- and 38-amino acid sequences, respectively. The TGs identified to date do not have a typical endoplasmic reticulum (ER)-signal peptide, and the molecular mechanisms of their secretion under physiologic conditions are unclear. Immunocytochemistry revealed that TG-A localizes to multivesicular-like structures, whereas TG-B localizes to the cytosol. We also found that TG-A, but not TG-B, was modified concomitantly by *N*-myristoylation and *S*-palmitoylation, and *N*-myristoylation was a pre-requisite for *S*-palmitoylation. Moreover, TG-A, but not TG-B, was secreted in response to calcium signaling induced by Ca^{2+} ionophores and uracil, a pathogenic bacteria-derived substance. Brefeldin A and monensin, inhibitors of the ER/Golgi-mediated conventional pathway, did not suppress TG-A secretion, whereas inhibition of *S*-palmitoylation by 2-bromopalmitate blocked TG-A secretion. Ultracentrifugation, electron microscopy analyses, and treatments with inhibitors of multivesicular body formation revealed that TG-A was secreted via exosomes together with co-transfected mammalian CD63, an exosomal marker, and the secreted TG-A was taken up by other cells. The 8-residue N-terminal fragment of TG-A containing the fatty acylation sites was both necessary and sufficient for the exosome-dependent secretion of TG-A. In conclusion, TG-A is secreted through an unconventional ER/Golgi-independent pathway involving two types of fatty acylations and exosomes.

3.2769 Contribution of neuroblastoma-derived exosomes to the production of pro-tumorigenic signals by bone marrow mesenchymal stromal cells
Nakata, R., Shimada, H., Fernandez, G.E., Fanter, R., Fabbri, M., malvar, J., Zimmermann, P. and DeClerk, Y.A.
*J. Extracellular Vesicles, **6**:1, 1332941 (2017)*

The bone marrow (BM) niche is a microenvironment promoting survival, dormancy and therapeutic resistance in tumor cells. Central to this function are mesenchymal stromal cells (MSCs). Here, using neuroblastoma (NB) as a model, we demonstrate that NB cells release an extracellular vesicle (EVs) whose protein cargo is enriched in exosomal proteins but lacks cytokines and chemokines. Using three different purification methods, we then demonstrate that NB-derived exosomes were captured by MSCs and induced the production of pro-tumorigenic cytokines and chemokines, including interleukin-6 (IL-6), IL-8/CXCL8, vascular endothelial cell growth factor and monocyte-chemotactic protein-1, with exosomes

prepared by size exclusion chromatography having the highest activity. We found no correlation between the IL-6 and IL-8/CXCL8 stimulatory activity of exosomes from eight NB cell lines and their origin, degree of MYCN amplification, drug resistance and disease status. We then demonstrate that the uptake of NB exosomes by MSCs was associated with a rapid increase in ERK1/2 and AKT activation, and that blocking ERK1/2 but not AKT activation inhibited the IL-6 and IL-8/CXCL8 production by MSCs without affecting exosome uptake. Thus, we describe a new mechanism by which NB cells induce in MSCs an inflammatory reaction that contributes to a favorable microenvironment in the BM.

3.2770 GSK3β and ERK regulate the expression of 78 kDa SG2NA and ectopic modulation of its level affects phases of cell cycle
Pandey, S., Talukdar, I., Jain, B.P. and Goswami, S.K.
*Scientific Reports, **7**, 7555 (2017)*

Striatin and SG2NA are essential constituents of the multi-protein STRIPAK assembly harbouring protein phosphatase PP2A and several kinases. SG2NA has several isoforms generated by mRNA splicing and editing. While the expression of striatin is largely restricted to the striatum in brain, that of SG2NAs is ubiquitous. In NIH3T3 cells, only the 78 kDa isoform is expressed. When cells enter into the S phase, the level of SG2NA increases; reaches maximum at the G2/M phase and declines thereafter. Downregulation of SG2NA extends G1 phase and its overexpression extends G2. Ectopic expression of the 35 kDa has no effects on the cell cycle. Relative abundance of phospho-SG2NA is high in the microsome and cytosol and the nucleus but low in the mitochondria. Okadoic acid, an inhibitor of PP2A, increases the level of SG2NA which is further enhanced upon inhibition of proteasomal activity. Phospho-SG2NA is thus more stable than the dephosphorylated form. Inhibition of GSK3β by LiCl reduces its level, but the inhibition of ERK by PD98059 increases it. Thus, ERK decreases the level of phospho-SG2NA by inhibiting GSK3β. In cells depleted from SG2NA by shRNA, the levels of pGSK3β and pERK are reduced, suggesting that these kinases and SG2NA regulate each other's expression.

3.2771 Isolation of mouse chromaffin secretory vesicles and their division into 12 fractions
Pardo, M.R., Estevez-Herrera, J., Castaneyra, L., Borges, R. and Machado, J.D.
*Anal. Biochem., **536**, 1-7 (2017)*

The study of chromaffin secretory vesicles (SVs) has contributed immensely to our understanding of exocytosis. These organelles, also called chromaffin granules, are a specific type of large dense secretory vesicle found in many endocrine cells and neurons. Traditionally, they have been isolated from bovine adrenal glands due to the large number of SVs that can be obtained from this tissue. However, technical advances now make it possible to obtain very pure preparations of SVs from mice, which is particular interesting for functional studies given the availability of different genetically modified strains of mice. Despite the small size of the mouse adrenal medulla (400–500 μm and less than 2 mg in weight), we have successfully carried out functional studies on SVs isolated from WT and knockout mice. As such, we present here our method to purify crude vesicles and to fractionate mouse chromaffin SVs, along with examples of their functional characterization.

3.2772 The iron chaperone poly(rC)-binding protein 2 forms a metabolon with the heme oxygenase 1/cytochrome P450 reductase complex for heme catabolism and iron transfer
Yanatori, I., Richaaardson, D.r., Toyokuni, S. and Kishi, F.
*J. Biol. Chem., **292(32)**, 13205-13229 (2017)*

Mammals incorporate a major proportion of absorbed iron as heme, which is catabolized by the heme oxygenase 1 (HO1)–NADPH-cytochrome P450 reductase (CPR) complex into biliverdin, carbon monoxide, and ferrous iron. Moreover, intestinal iron is incorporated as ferrous iron, which is transported via the iron importer, divalent metal transporter 1 (DMT1). Recently, we demonstrated that the iron chaperone poly(rC)-binding protein 2 (PCBP2) can directly receive ferrous iron from DMT1 or transfer iron to the iron exporter, ferroportin 1. To promote intracellular iron flux, an iron chaperone may be essential for receiving iron generated by heme catabolism, but this hypothesis is untested so far. Herein, we demonstrate that HO1 binds to PCBP2, but not to other PCBP family members, namely PCBP1, PCBP3, or PCBP4. Interestingly, HO1 formed a complex with either CPR or PCBP2, and it was demonstrated that PCBP2 competes with CPR for HO1 binding. Using PCBP2-deletion mutants, we demonstrated that the PCBP2 K homology 3 domain is important for the HO1/PCBP2 interaction. In heme-loaded cells, heme prompted HO1–CPR complex formation and decreased the HO1/PCBP2 interaction. Furthermore, *in vitro* reconstitution experiments with purified recombinant proteins indicated that HO1 could bind to PCBP2 in

the presence of heme, whereas loading of PCBP2 with ferrous iron caused PCBP2 to lose its affinity for HO1. These results indicate that ferrous iron released from heme can be bound by PCBP2 and suggest a model for an integrated heme catabolism and iron transport metabolon.

3.2773 Serum-derived extracellular vesicles (EVs) impact on vascular remodeling and prevent muscle damage in acute hind limb ischemia
Cavallari, C., Ranghino, A., Tapparo, M., Cedrino, M., Figliolini, F., Grange, C., Giannachi, V., Garneri, P., Deregibus, M.C., Collino, F., Rispoli, P., Camussi, G. and Brizzi, M.F.
Scientific Reports, 7:8180 (2017)

Serum is an abundant and accessible source of circulating extracellular vesicles (EVs). Serum-EV (sEV) pro-angiogenic capability and mechanisms are herein analyzed using an *in vitro* assay which predicts sEV angiogenic potential *in vivo*. Effective sEVs (e-sEVs) also improved vascular remodeling and prevented muscle damage in a mouse model of acute hind limb ischemia. e-sEV angiogenic proteomic and transcriptomic analyses show a positive correlation with matrix-metalloproteinase activation and extracellular matrix organization, cytokine and chemokine signaling pathways, Insulin-like Growth Factor and platelet pathways, and Vascular Endothelial Growth Factor signaling. A discrete gene signature, which highlights differences in e-sEV and ineffective-EV biological activity, was identified using gene ontology (GO) functional analysis. An enrichment of genes associated with the Transforming Growth Factor beta 1 (TGFβ1) signaling cascade is associated with e-sEV administration but not with ineffective-EVs. Chromatin immunoprecipitation analysis on the *inhibitor of DNA binding I* (ID1) promoter region, and the knock-down of *small mother against decapentaplegic* (SMAD)1–5 proteins confirmed GO functional analyses. This study demonstrates sEV pro-angiogenic activity, validates a simple, sEV pro-angiogenic assay which predicts their biological activity *in vivo*, and identifies the TGFβ1 cascade as a relevant mediator. We propose serum as a readily available source of EVs for therapeutic purposes.

3.2774 Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated DNA
Nemeth, A. et al
Scientific Reports, 7:8202 (2017)

Recently, biological roles of extracellular vesicles (which include among others exosomes, microvesicles and apoptotic bodies) have attracted substantial attention in various fields of biomedicine. Here we investigated the impact of sustained exposure of cells to the fluoroquinolone antibiotic ciprofloxacin on the released extracellular vesicles. Ciprofloxacin is widely used in humans against bacterial infections as well as in cell cultures against *Mycoplasma* contamination. However, ciprofloxacin is an inducer of oxidative stress and mitochondrial dysfunction of mammalian cells. Unexpectedly, here we found that ciprofloxacin induced the release of both DNA (mitochondrial and chromosomal sequences) and DNA-binding proteins on the exofacial surfaces of small extracellular vesicles referred to in this paper as exosomes. Furthermore, a label-free optical biosensor analysis revealed DNA-dependent binding of exosomes to fibronectin. DNA release on the surface of exosomes was not affected any further by cellular activation or apoptosis induction. Our results reveal for the first time that prolonged low-dose ciprofloxacin exposure leads to the release of DNA associated with the external surface of exosomes.

3.2775 Generation and intracellular trafficking of a polysialic acid-carrying fragment of the neural cell adhesion molecule NCAM to the cell nucleus
Westphal, N., Loers, G., Lutz, D., Theis, T., Kleene, R. and Schachner, M.
Scientific Reports, 7:8622 (2017)

Polysialic acid (PSA) and its major protein carrier, the neural cell adhesion molecule NCAM, play important roles in many nervous system functions during development and in adulthood. Here, we show that a PSA-carrying NCAM fragment is generated at the plasma membrane by matrix metalloproteases and transferred to the cell nucleus via endosomes and the cytoplasm. Generation and nuclear import of this fragment in cultured cerebellar neurons is induced by a function-triggering NCAM antibody and a peptide comprising the effector domain (ED) of myristoylated alanine-rich C kinase substrate (MARCKS) which interacts with PSA within the plane of the plasma membrane. These treatments lead to activation of the fibroblast growth factor (FGF) receptor, phospholipase C (PLC), protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K), and subsequently to phosphorylation of MARCKS. Moreover, the NCAM antibody triggers calmodulin-dependent activation of nitric oxide synthase, nitric oxide (NO) production, NO-dependent S-nitrosylation of matrix metalloprotease 9 (MMP9) as well as activation of matrix metalloprotease 2 (MMP2) and MMP9, whereas the ED peptide activates phospholipase D (PLD)

and MMP2, but not MMP9. These results indicate that the nuclear PSA-carrying NCAM fragment is generated by distinct and functionally defined signal transducing mechanisms.

3.2776 Regulated Polarization of Tumor-Associated Macrophages by miR-145 via Colorectal Cancer–Derived Extracellular Vesicles

Shinohara, H., Kuranaga, Y., Kumazaki, M., Sugito, N., Yoshikawa, Y., Takai, T., Taniguchi, K., Ito, Y. and Akao, Y.
J. Immunol., ***199(4)***, *1505-1515 (2017)*

Macrophages are polarized into functional classically activated and alternatively activated (M2) phenotypes depending on their microenvironment, and these cells play an important role in the immune system. M2-like polarization of tumor-associated macrophages (TAMs) is activated by various secretions from cancer cells; however, the interaction between cancer cells and TAMs is not well understood. Recent studies showed that cancer cell–derived extracellular vesicles (EVs) contribute to tumor development and modulation of the tumor microenvironment. In the current study, we investigated colorectal cancer–derived EVs containing miR-145 with respect to the polarization of TAMs. Colorectal cancer cells positively secreted miR-145 via EVs, which were taken up by macrophage-like cells. Interestingly, colorectal cancer–derived EVs polarized macrophage-like cells into the M2-like phenotype through the downregulation of *histone deacetylase 11*. An in vivo study showed that EV-treated macrophages caused significant enlargement of the tumor volumes. These findings suggest that colorectal cancer cells use miR-145 within EVs to efficiently modulate M2-like macrophage polarization and tumor progression.

3.2777 Surface LAMP-2 Is an Endocytic Receptor That Diverts Antigen Internalized by Human Dendritic Cells into Highly Immunogenic Exosomes

Leone, D.A., Peschel, A., Brown, M., Schachner, H., Ball, M.J., Gyuraszova, M., Salzer-Muhar, U.
J. Immunol., ***199(2)***, *531-546 (2017)*

The lysosome-associated membrane protein (LAMP) family includes the dendritic cell endocytic receptors DC-LAMP and CD68, as well as LAMP-1 and LAMP-2. In this study we identify LAMP-1 (CD107a) and LAMP-2 (CD107b) on the surface of human monocyte-derived dendritic cells (MoDC) and show only LAMP-2 is internalized after ligation by specific Abs, including H4B4, and traffics rapidly but transiently to the MHC class II loading compartment, as does Ag conjugated to H4B4. However, pulsing MoDC with conjugates of primary (keyhole limpet hemocyanin; KLH) and recall (Bet v 1) Ags (H4B4*KLH and H4B4*Bet v 1) induced significantly less CD4 cell proliferation than pulsing with native Ag or Ag conjugated to control mAb (ISO*KLH and ISO*Bet v 1). In H4B4*KLH-pulsed MoDC, the duration of KLH residence in MHC class II loading compartments was significantly reduced, as were surface HLA-DR and DR-bound KLH-derived peptides. Paradoxically, MoDC pulsed with H4B4*KLH, but not the other KLH preparations, induced robust proliferation of CD4 cells separated from them by a transwell membrane, indicating factors in the supernatant were responsible. Furthermore, extracellular vesicles from supernatants of H4B4*KLH-pulsed MoDC contained significantly more HLA-DR and KLH than those purified from control MoDC, and KLH was concentrated specifically in exosomes that were a uniquely effective source of Ag in standard T cell proliferation assays. In summary, we identify LAMP-2 as an endocytic receptor on human MoDC that routes cargo into unusual Ag processing pathways, which reduces surface expression of Ag-derived peptides while selectively enriching Ag within immunogenic exosomes. This novel pathway has implications for the initiation of immune responses both locally and at distant sites.

3.2778 Caspase-8 controls the secretion of inflammatory lysyl-tRNA synthetase in exosomes from cancer cells

Kim, S.B. et al
J. Cell Biol.,***216(7)***, *2201-2216 (2017)*

Aminoacyl-tRNA synthetases (ARSs), enzymes that normally control protein synthesis, can be secreted and have different activities in the extracellular space, but the mechanism of their secretion is not understood. This study describes the secretion route of the ARS lysyl-tRNA synthetase (KRS) and how this process is regulated by caspase activity, which has been implicated in the unconventional secretion of other proteins. We show that KRS is secreted from colorectal carcinoma cells within the lumen of exosomes that can trigger an inflammatory response. Caspase-8 cleaved the N-terminal of KRS, thus exposing a PDZ-binding motif located in the C terminus of KRS. Syntenin bound to the exposed PDZ-binding motif of KRS and facilitated the exosomic secretion of KRS dissociated from the multi-tRNA

synthetase complex. KRS-containing exosomes released by cancer cells induced macrophage migration, and their secretion of TNF-α and cleaved KRS made a significant contribution to these activities, which suggests a novel mechanism by which caspase-8 may promote inflammation.

3.2779 Acinetobacter baumannii transfers the blaNDM-1 gene via outer membrane vesicles
Chatterjee, S., Mondal, A., Mitra, S. and Basu, S.
J. Antimicrob. Chemother., ***72(8)***, *2201-2207 (2017)*

Objectives: To investigate the transmission of the gene encoding New Delhi metallo-β-lactamase-1 (bla_{NDM-1}) through outer membrane vesicles (OMVs) released from an *Acinetobacter baumannii* strain (A_115).
Methods: Isolation and purification of OMVs by density gradient from a carbapenem-resistant clinical strain of *A. baumannii* harbouring plasmid-mediated bla_{NDM-1} and *aac(6')-Ib-cr* genes was performed. DNA was purified from the OMVs and used for PCR and dot-blot analysis. Vesicles treated with DNase I and proteinase K were used to transform *A. baumannii* ATCC 19606 and *Escherichia coli* JM109 strains. MIC values for the transformants were determined, followed by PCR and restriction digestion of plasmids. PFGE was done for A_115 and transformants of ATCC 19606 and JM109.
Results: The *A. baumannii* strain (ST 1462) released vesicles (25–100 nm) during *in vitro* growth at late log phase. PCR and dot-blot analysis confirmed the presence of bla_{NDM-1} and *aac(6')-Ib-cr* genes in intravesicular DNA. bla_{NDM-1} and *aac(6')-Ib-cr* genes were transferred to both the *A. baumannii* ATCC 19606 and *E. coli* JM109 recipient cells. The transformation frequency of the purified OMVs was in the range of 10^{-5}–10^{-6} and gradually reduced with storage of OMVs. The sizes of the plasmids in the transformants and their restriction digestion patterns were identical to the plasmid in A_115. The transformants showed elevated MIC values of the β-lactam group of antibiotics, which confirmed the presence of a bla_{NDM-1}-harbouring plasmid.
Conclusions: This is the first experimental evidence of intra- and inter-species transfer of a plasmid harbouring a bla_{NDM-1} gene in *A. baumannii* via OMVs with high transformation frequency.

3.2780 Binding of canonical Wnt ligands to their receptor complexes occurs in ordered plasma membrane environments
Sezgin, E., Azbazdar, Y., Ng, X.W., The, C., Simons, K., Weidinger, G., Wohland, T., Eggeling, C. and Ozhan, G.
FEBS J., ***284(15)***, *2513-2526 (2017)*

While the cytosolic events of Wnt/β-catenin signaling (canonical Wnt signaling) pathway have been widely studied, only little is known about the molecular mechanisms involved in Wnt binding to its receptors at the plasma membrane. Here, we reveal the influence of the immediate plasma membrane environment on the canonical Wnt–receptor interaction. While the receptors are distributed both in ordered and disordered environments, Wnt binding to its receptors selectively occurs in more ordered membrane environments which appear to cointernalize with the Wnt-receptor complex. Moreover, Wnt/β-catenin signaling is significantly reduced when the membrane order is disturbed by specific inhibitors of certain lipids that prefer to localize at the ordered environments. Similarly, a reduction in Wnt signaling activity is observed in Niemann–Pick Type C disease cells where trafficking of ordered membrane lipid components to the plasma membrane is genetically impaired. We thus conclude that ordered plasma membrane environments are essential for binding of canonical Wnts to their receptor complexes and downstream signaling activity.

3.2781 S100-A9 protein in exosomes from chronic lymphocytic leukemia cells promotes NF-κB activity during disease progression
Prieto, D., Sotelo, N., Seija, N., Sernbo, S., Abreu, C., Duran, R., Gil, M., Sicco, E., irigoin, V., Oliver, C., Landoni, A.I., gabus, R., Dighiero, G. and Opezzo, P.
Blood, ***130(6)***, *777-788 (2017)*

Chronic lymphocytic leukemia (CLL) is an incurable disease characterized by accumulation of clonal B lymphocytes, resulting from a complex balance between cell proliferation and apoptotic death. Continuous crosstalk between cancer cells and local/distant host environment is required for effective tumor growth. Among the main actors of this dynamic interplay between tumoral cells and their microenvironment are the nano-sized vesicles called exosomes. Emerging evidence indicates that secretion, composition, and functional capacity of exosomes are altered as tumors progress to an aggressive phenotype. In CLL, no data exist exploring the specific changes in the proteomic profile of plasma-derived exosomes from

patients during disease evolution. We hereby report for the first time different proteomic profiles of plasma exosomes, both between indolent and progressive CLLs as well as within the individual patients at the onset of disease and during its progression. Next, we focus on the changes of the exosome protein cargoes, which are found exclusively in patients with progressive CLL after disease progression. The alterations in the proteomic cargoes underline different networks specific for leukemia progression related to inflammation, oxidative stress, and NF-κB and phosphatidylinositol 3-kinase/AKT pathway activation. Finally, our results suggest a preponderant role for the protein S100-A9 as an activator of the NFκB pathway during CLL progression and suggest that the leukemic clone can generate an autoactivation loop through S100-A9 expression, NF-κB activation, and exosome secretion. Collectively, our data propose a new pathway for NF-κB activation in CLL and highlight the importance of exosomes as extracellular mediators promoting tumor progression in CLL.

3.2782 A complex of Neuroplastin and Plasma Membrane Ca2+ ATPase controls T cell activation
Korthals, M. et al
Scientific Reports, 7:8358 (2017)

The outcome of T cell activation is determined by mechanisms that balance Ca^{2+} influx and clearance. Here we report that murine CD4 T cells lacking Neuroplastin (*Nptn*$^{-/-}$), an immunoglobulin superfamily protein, display elevated cytosolic Ca^{2+} and impaired post-stimulation Ca^{2+} clearance, along with increased nuclear levels of NFAT transcription factor and enhanced T cell receptor-induced cytokine production. On the molecular level, we identified plasma membrane Ca^{2+} ATPases (PMCAs) as the main interaction partners of Neuroplastin. PMCA levels were reduced by over 70% in *Nptn*$^{-/-}$ T cells, suggesting an explanation for altered Ca^{2+} handling. Supporting this, Ca^{2+} extrusion was impaired while Ca^{2+} levels in internal stores were increased. T cells heterozygous for PMCA1 mimicked the phenotype of *Nptn*$^{-/-}$ T cells. Consistent with sustained Ca^{2+} levels, differentiation of *Nptn*$^{-/-}$ T helper cells was biased towards the Th1 versus Th2 subset. Our study thus establishes Neuroplastin-PMCA modules as important regulators of T cell activation.

3.2783 Degradation of cofilin is regulated by Cbl, AIP4 and Syk resulting in increased migration of LMP2A positive nasopharyngeal carcinoma cells
Gainullin, M.R., Zhukov, I.Y., Zhou, X., Mo, Y., Astakhova, L., Ernberg, I. and Matskova, L.
Scientific Reports, 7:9012 (2017)

Expression of cofilin is directly associated with metastatic activity in many tumors. Here, we studied the role of Latent Membrane Protein 2 A (LMP2A) of Epstein-Barr Virus (EBV) in the accumulation of cofilin observed in nasopharyngeal cancer (NPC) tumor cells. We used LMP2A transformed NPC cell lines to analyze cofilin expression. We used mutation analysis, ectopic expression and down-regulation of Cbl, AIP4 and Syk in these cell lines to determine the effect of the LMP2A viral protein on cofilin degradation and its role in the assembly of a cofilin degrading protein complex. The LMP2A of EBV was found to interfer with cofilin degradation in NPC cells by accelerating the proteasomal degradation of Cbl and Syk. In line with this, we found significantly higher cofilin expression in NPC tumor samples as compared to the surrounding epithelial tissues. Cofilin, as an actin severing protein, influences cellular plasticity, and facilitates cellular movement in response to oncogenic stimuli. Thus, under relaxed cellular control, cofilin facilitates tumor cell movement and dissemination. Interference with its degradation may enhance the metastatic potential of NPC cells.

3.2784 Sterol targeting drugs reveal life cycle stage-specific differences in trypanosome lipid rafts
Sharma, A.I., Olson, C.L., Marmede, J.I., Gazos-Lopes, F., Epting, C.L., Almeida, I.C. and Engman, D.M.
Scientific Reports, 7:9105 (2017)

Cilia play important roles in cell signaling, facilitated by the unique lipid environment of a ciliary membrane containing high concentrations of sterol-rich lipid rafts. The African trypanosome *Trypanosoma brucei* is a single-celled eukaryote with a single cilium/flagellum. We tested whether flagellar sterol enrichment results from selective flagellar partitioning of specific sterol species or from general enrichment of all sterols. While all sterols are enriched in the flagellum, cholesterol is especially enriched. *T. brucei* cycles between its mammalian host (bloodstream cell), in which it scavenges cholesterol, and its tsetse fly host (procyclic cell), in which it both scavenges cholesterol and synthesizes ergosterol. We wondered whether the insect and mammalian life cycle stages possess chemically different lipid rafts due to different sterol utilization. Treatment of bloodstream parasites with cholesterol-specific methyl-β-cyclodextrin disrupts both membrane liquid order and localization of a raft-associated ciliary membrane

calcium sensor. Treatment with ergosterol-specific amphotericin B does not. The opposite results were observed with ergosterol-rich procyclic cells. Further, these agents have opposite effects on flagellar sterol enrichment and cell metabolism in the two life cycle stages. These findings illuminate differences in the lipid rafts of an organism employing life cycle-specific sterols and have implications for treatment.

3.2785 Phosphatidylserine externalization, "necroptotic bodies" release, and phagocytosis during necroptosis

Zargarian, S., Shlomovitz, I., Erlich, Z., Hourizadeh, A., Ofir-Birin, Y.O., Croker, B.A., Regev-Rudzki, N., Edry-Botzer, L. and Gerlic, M.
*PloS Biology, **15(6)**, e2002711 (2017)*

Necroptosis is a regulated, nonapoptotic form of cell death initiated by receptor-interacting protein kinase-3 (RIPK3) and mixed lineage kinase domain-like (MLKL) proteins. It is considered to be a form of regulated necrosis, and, by lacking the "find me" and "eat me" signals that are a feature of apoptosis, necroptosis is considered to be inflammatory. One such "eat me" signal observed during apoptosis is the exposure of phosphatidylserine (PS) on the outer plasma membrane. Here, we demonstrate that necroptotic cells also expose PS after phosphorylated mixed lineage kinase-like (pMLKL) translocation to the membrane. Necroptotic cells that expose PS release extracellular vesicles containing proteins and pMLKL to their surroundings. Furthermore, inhibition of pMLKL after PS exposure can reverse the process of necroptosis and restore cell viability. Finally, externalization of PS by necroptotic cells drives recognition and phagocytosis, and this may limit the inflammatory response to this nonapoptotic form of cell death. The exposure of PS to the outer membrane and to extracellular vesicles is therefore a feature of necroptotic cell death and may serve to provide an immunologically-silent window by generating specific "find me" and "eat me" signals.

3.2786 Mitochondrial metabolic regulation by GRP78

Prasad, M., Pawlak, K.J., Burak, W.E., Perry, E.E., Marshall, B., Whittal, R.M. and Bose, H.S.
*Sci. Adv., **3**, e1602038 (2017)*

Steroids, essential for mammalian survival, are initiated by cholesterol transport by steroidogenic acute regulatory protein (StAR). Appropriate protein folding is an essential requirement of activity. Endoplasmic reticulum (ER) chaperones assist in folding of cytoplasmic proteins, whereas mitochondrial chaperones fold only mitochondrial proteins. We show that glucose regulatory protein 78 (GRP78), a master ER chaperone, is also present at the mitochondria-associated ER membrane (MAM), where it folds StAR for delivery to the outer mitochondrial membrane. StAR expression and activity are drastically reduced following GRP78 knockdown. StAR folding starts at the MAM region; thus, its cholesterol fostering capacity is regulated by GRP78 long before StAR reaches the mitochondria. In summary, GRP78 is an acute regulator of steroidogenesis at the MAM, regulating the intermediate folding of StAR that is crucial for its activity.

3.2787 Reconstitution of calcium-mediated exocytosis of dense-core vesicles

Kreutzberger, A.J.B., Kiessling, V., Linag, B., Seelheim, P., Jakhanwal, S., Jahn, R., Castle, J.D. and Tamm, L.K.
*Sci.Adv., **3**, e1603208 (2017)*

Regulated exocytosis is a process by which neurotransmitters, hormones, and secretory proteins are released from the cell in response to elevated levels of calcium. In cells, secretory vesicles are targeted to the plasma membrane, where they dock, undergo priming, and then fuse with the plasma membrane in response to calcium. The specific roles of essential proteins and how calcium regulates progression through these sequential steps are currently incompletely resolved. We have used purified neuroendocrine dense-core vesicles and artificial membranes to reconstruct in vitro the serial events that mimic SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor)–dependent membrane docking and fusion during exocytosis. Calcium recruits these vesicles to the target membrane aided by the protein CAPS (calcium-dependent activator protein for secretion), whereas synaptotagmin catalyzes calcium-dependent fusion; both processes are dependent on phosphatidylinositol 4,5-bisphosphate. The soluble proteins Munc18 and complexin-1 are necessary to arrest vesicles in a docked state in the absence of calcium, whereas CAPS and/or Munc13 are involved in priming the system for an efficient fusion reaction.

3.2788 MYC Mediates Large Oncosome-Induced Fibroblast Reprogramming in Prostate Cancer

Minciacchi, V.R: et al

Cancer Res., ***77(9)***, *2306-2317 (2017)*

Communication between cancer cells and the tumor microenvironment results in the modulation of complex signaling networks that facilitate tumor progression. Here, we describe a new mechanism of intercellular communication originating from large oncosomes (LO), which are cancer cell–derived, atypically large (1–10 μm) extracellular vesicles (EV). We demonstrate that, in the context of prostate cancer, LO harbor sustained AKT1 kinase activity, nominating them as active signaling platforms. Active AKT1 was detected in circulating EV from the plasma of metastatic prostate cancer patients and was LO specific. LO internalization induced reprogramming of human normal prostate fibroblasts as reflected by high levels of α-SMA, IL6, and MMP9. In turn, LO-reprogrammed normal prostate fibroblasts stimulated endothelial tube formation *in vitro* and promoted tumor growth in mice. Activation of stromal MYC was critical for this reprogramming and for the sustained cellular responses elicited by LO, both *in vitro* and *in vivo* in an AKT1-dependent manner. Inhibition of LO internalization prevented activation of MYC and impaired the tumor-supporting properties of fibroblasts. Overall, our data show that prostate cancer–derived LO powerfully promote establishment of a tumor-supportive environment by inducing a novel reprogramming of the stroma. This mechanism offers potential alternative options for patient treatment.

3.2789 **Sigma1 Targeting to Suppress Aberrant Androgen Receptor Signaling in Prostate Cancer**
Thomas, J.D., Longen, C.G., Oyer, H.M., Chen, N., Maher, C.M., Salvino, J.M., Kania, B., Anderson, K.N., Ostrander, W.F., Knudsen, K.E. and Kim, F.J.
Cancer Res.,***77(9)***, *2439-2452 (2017)*

Suppression of androgen receptor (AR) activity in prostate cancer by androgen depletion or direct AR antagonist treatment, although initially effective, leads to incurable castration-resistant prostate cancer (CRPC) via compensatory mechanisms including resurgence of AR and AR splice variant (ARV) signaling. Emerging evidence suggests that Sigma1 (also known as sigma-1 receptor) is a unique chaperone or scaffolding protein that contributes to cellular protein homeostasis. We reported previously that some Sigma1-selective small molecules can be used to pharmacologically modulate protein homeostasis pathways. We hypothesized that these Sigma1-mediated responses could be exploited to suppress AR protein levels and activity. Here we demonstrate that treatment with a small-molecule Sigma1 inhibitor prevented 5α- dihydrotestosterone-mediated nuclear translocation of AR and induced proteasomal degradation of AR and ARV, suppressing the transcriptional activity and protein levels of both full-length and splice-variant AR. Consistent with these data, RNAi knockdown of Sigma1 resulted in decreased AR levels and transcriptional activity. Furthermore, Sigma1 physically associated with ARV7 and AR^{v567es} as well as full-length AR. Treatment of mice xenografted with ARV-driven CRPC tumors with a drug-like small-molecule Sigma1 inhibitor significantly inhibited tumor growth associated with elimination of AR and ARV7 in responsive tumors. Together, our data show that Sigma1 modulators can be used to suppress AR/ARV–driven prostate cancer cells via regulation of pharmacologically responsive Sigma1-AR/ARV interactions, both in vitro and in vivo.

3.2790 **Differential Protein Expression Marks the Transition From Infection With Opisthorchis viverrini to Cholangiocarcinoma**
Khoontawad, J., Pairojkul, C., Rucksaken, R., Pinlaor, P., WOngkham, C., Yongvanit, P., Pugkhem, A., Jones, A., Plieskatt, J., Potriquet, J., Bethony, J., Pinlaor, S. and Mulvenna, J.
Mol. Cell. Proteomics, ***16(5)***, *911-923 (2017)*

Parts of Southeast Asia have the highest incidence of intrahepatic cholangiocarcinoma (CCA) in the world because of infection by the liver fluke *Opisthorchis viverrini* (Ov). Ov-associated CCA is the culmination of chronic Ov-infection, with the persistent production of the growth factors and cytokines associated with persistent inflammation, which can endure for years in Ov-infected individuals prior to transitioning to CCA. Isobaric labeling and tandem mass spectrometry of liver tissue from a hamster model of CCA was used to compare protein expression profiles from inflammed tissue (Ovinfected but not cancerous) *versus* cancerous tissue (Ov-induced CCA). Immunohistochemistry and immunoblotting were used to verify dysregulated proteins in the animal model and in human tissue. We identified 154 dysregulated proteins that marked the transition from Ov-infection to Ov-induced CCA, *i.e.* proteins dysregulated during carcinogenesis but not Ov-infection. The verification of dysregulated proteins in resected liver tissue from humans with Ov-associated CCA showed the numerous parallels in protein dysregulation between human and animal models of Ov-induced CCA. To identify potential circulating markers for CCA, dysregulated proteins were compared with proteins isolated from exosomes secreted by a human CCA cell line (KKU055) and 27 proteins were identified as dysregulated in CCA and present in exosomes. These data

form the basis of potential diagnostic biomarkers for human Ov-associated CCA. The profile of protein dysregulation observed during chronic Ovinfection and then in Ov-induced CCA provides insight into the etiology of an infection-induced inflammation-related cancer.

3.2791 Retrograde trafficking of β-dystroglycan from the plasma membrane to the nucleus
Gracida-Jimenez, V., Mondragon-Gonzalez, R., Velez-Aguilera, G., Vasquez, A., Laredo-Cisneros, M.S., de Dios Gomez-Lopez, J., Vaca, L., Gourlay, S.C., Jacobs, L.A., Winder, S.J. and Cisneros, B.
*Scientific Reports, **7**:9906 (2017)*

β-Dystroglycan (β-DG) is a transmembrane protein with critical roles in cell adhesion, cytoskeleton remodeling and nuclear architecture. This functional diversity is attributed to the ability of β-DG to target to, and conform specific protein assemblies at the plasma membrane (PM) and nuclear envelope (NE). Although a classical NLS and importin α/β mediated nuclear import pathway has already been described for β-DG, the intracellular trafficking route by which β-DG reaches the nucleus is unknown. In this study, we demonstrated that β-DG undergoes retrograde intracellular trafficking from the PM to the nucleus via the endosome-ER network. Furthermore, we provided evidence indicating that the translocon complex Sec61 mediates the release of β-DG from the ER membrane, making it accessible for importins and nuclear import. Finally, we show that phosphorylation of β-DG at Tyr890 is a key stimulus for β-DG nuclear translocation. Collectively our data describe the retrograde intracellular trafficking route that β-DG follows from PM to the nucleus. This dual role for a cell adhesion receptor permits the cell to functionally connect the PM with the nucleus and represents to our knowledge the first example of a cell adhesion receptor exhibiting retrograde nuclear trafficking and having dual roles in PM and NE.

3.2792 New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes
Fernando, M.R., Jiang, C., Krzyanowski, G.D. and Ryan, W.L.
*PloS One, **12(8)**, e0183915 (2017)*

Cell-free DNA (cfDNA) in blood is used as a source of genetic material for noninvasive prenatal and cancer diagnostic assays in clinical practice. Recently we have started a project for new biomarker discovery with a view to developing new noninvasive diagnostic assays. While reviewing literature, it was found that exosomes may be a rich source of biomarkers, because exosomes play an important role in human health and disease. While characterizing exosomes found in human blood plasma, we observed the presence of cfDNA in plasma exosomes. Plasma was obtained from blood drawn into K_3EDTA tubes. Exosomes were isolated from cell-free plasma using a commercially available kit. Sizing and enumeration of exosomes were done using electron microscopy and NanoSight particle counter. NanoSight and confocal microscopy was used to demonstrate the association between dsDNA and exosomes. DNA extracted from plasma and exosomes was measured by a fluorometric method and a droplet digital PCR (ddPCR) method. Size of extracellular vesicles isolated from plasma was heterogeneous and showed a mean value of 92.6 nm and a mode 39.7 nm. A large proportion of extracellular vesicles isolated from plasma were identified as exosomes using a fluorescence probe specific for exosomes and three protein markers, Hsp70, CD9 and CD63, that are commonly used to identify exosome fraction. Fluorescence dye that stain dsDNA showed the association between exosomes and dsDNA. Plasma cfDNA concentration analysis showed more than 93% of amplifiable cfDNA in plasma is located in plasma exosomes. Storage of a blood sample showed significant increases in exosome count and exosome DNA concentration. This study provide evidence that a large proportion of plasma cfDNA is localized in exosomes. Exosome release from cells is a metabolic energy dependent process, thus suggesting active release of cfDNA from cells as a source of cfDNA in plasma.

3.2793 Tylophorine Analogs Allosterically Regulates Heat Shock Cognate Protein 70 And Inhibits Hepatitis C Virus Replication
Wang, Y. et al
*Scientific Reports, **7**:10037 (2017)*

Tylophorine analogs have been shown to exhibit diverse activities against cancer, inflammation, arthritis, and lupus *in vivo*. In this study, we demonstrated that two tylophorine analogs, DCB-3503 and *rac*-cryptopleurine, exhibit potent inhibitory activity against hepatitis C virus (HCV) replication in genotype 1b Con 1 isolate. The inhibition of HCV replication is at least partially mediated through cellular heat shock cognate protein 70 (Hsc70). Hsc70 associates with the HCV replication complex by primarily binding to the poly U/UC motifs in HCV RNA. The interaction of DCB-3503 and *rac*-cryptopleurine with Hsc70 promotes the ATP hydrolysis activity of Hsc70 in the presence of the 3′ poly U/UC motif of HCV RNA.

Regulating the ATPase activity of Hsc70 may be one of the mechanisms by which tylophorine analogs inhibit HCV replication. This study demonstrates the novel anti-HCV activity of tylophorine analogs. Our results also highlight the importance of Hsc70 in HCV replication.

3.2794 Concise Review: Developing Best-Practice Models for the Therapeutic Use of Extracellular Vesicles
Reiner, A.T. et al
*Stem Cell Trans. Med., **6**, 1730-1739 (2017)*

Growing interest in extracellular vesicles (EVs, including exosomes and microvesicles) as therapeutic entities, particularly in stem cell-related approaches, has underlined the need for standardization and coordination of development efforts. Members of the International Society for Extracellular Vesicles and the Society for Clinical Research and Translation of Extracellular Vesicles Singapore convened a Workshop on this topic to discuss the opportunities and challenges associated with development of EV-based therapeutics at the preclinical and clinical levels. This review outlines topic-specific action items that, if addressed, will enhance the development of best-practice models for EV therapies.

3.2795 The Prohormone VGF Regulates β Cell Function via Insulin Secretory Granule Biogenesis
Stephens, S.B., Edwards, R.J., Sadahiro, M., Lin, W-J., Jiang, C., Salton, S.R. and Newgard, C.B.
*Cell Reports, **20**, 2480-2489 (2017)*

The prohormone VGF is expressed in neuroendocrine and endocrine tissues and regulates nutrient and energy status both centrally and peripherally. We and others have shown that VGF-derived peptides have direct action on the islet β cell as secretagogues and cytoprotective agents; however, the endogenous function of VGF in the β cell has not been described. Here, we demonstrate that VGF regulates secretory granule formation. VGF loss-of-function studies in both isolated islets and conditional knockout mice reveal a profound decrease in stimulus-coupled insulin secretion. Moreover, VGF is necessary to facilitate efficient exit of granule cargo from the *trans*-Golgi network and proinsulin processing. It also functions to replenish insulin granule stores following nutrient stimulation. Our data support a model in which VGF operates at a critical node of granule biogenesis in the islet β cell to coordinate insulin biosynthesis with β cell secretory capacity.

3.2796 Prophage-triggered membrane vesicle formation through peptidoglycan damage in Bacillus subtilis
Toyofuku, M., Carcamo-Oyarce, G., Yamamoto, T., Eisenstein, F., Hsiao, C-C., Kurosawa, M., Gademann, K., Pilhofer, M., Nomura, N. and Eberl, L.
*Nature Communications, **8**:481 (2017)*

Bacteria release membrane vesicles (MVs) that play important roles in various biological processes. However, the mechanisms of MV formation in Gram-positive bacteria are unclear, as these cells possess a single cytoplasmic membrane that is surrounded by a thick cell wall. Here we use live cell imaging and electron cryo-tomography to describe a mechanism for MV formation in *Bacillus subtilis*. We show that the expression of a prophage-encoded endolysin in a sub-population of cells generates holes in the peptidoglycan cell wall. Through these openings, cytoplasmic membrane material protrudes into the extracellular space and is released as MVs. Due to the loss of membrane integrity, the induced cells eventually die. The vesicle-producing cells induce MV formation in neighboring cells by the enzymatic action of the released endolysin. Our results support the idea that endolysins may be important for MV formation in bacteria, and this mechanism may potentially be useful for the production of MVs for applications in biomedicine and nanotechnology.

3.2797 Exosomes expressing carbonic anhydrase 9 promote angiogenesis
Horie, K., Kawakami, K., Fujita, Y., Sugaya, M., Kameyama, K., Mizutani, K., Deguchi, T. and Ito, M.
*Biochem. Biophys. Res. Comm., **492**, 356-361 (2017)*

Exosomes or microvesicles that are secreted from cells are considered to play important roles in tumor microenvironment. Carbonic anhydrase 9 (CA9), which is induced by hypoxia-inducible factor 1 (HIF1) in response to hypoxia, is overexpressed in many types of cancer including renal cell carcinoma (RCC). We examined the expression level of CA9 in several RCC cell lines and found that the basal level of CA9 was much higher in OSRC-2 cells than in Caki-1, KMRC-1 and 786-O cells. Consistent with the intracellular expression levels, CA9 was abundantly detected in exosomes isolated by ultracentrifugation from OSRC-2 cells. Density gradient centrifugation of OSRC-2 and 786-O exosomes confirmed the co-presence of CA9 with exosomal markers. Upon hypoxia and treatment with $CoCl_2$, a hypoxia mimic agent, the CA9

level in exosomes was increased for all cell lines. In order to examine the effects of CA9 exosomes on angiogenesis, we generated stably transfected HEK293 cells expressing CA9. Immunocytochemical staining demonstrated the uptake of CA9 exosomes by human umbilical vein endothelial cells (HUVEC). *In vitro* angiogenesis assays using HUVEC revealed that CA9 exosomes promoted migration and tube formation. Lastly, MMP2 expression was increased by treatment with CA9 exosomes in HUVEC. Taken together, our results suggest the possibility that CA9 exosomes released from hypoxic RCC may enhance angiogenesis in microenvironment, thereby contributing to cancer progression.

3.2798 Excess Translation of Epigenetic Regulators Contributes to Fragile X Syndrome and Is Alleviated by Brd4 Inhibition
Korb, E., Herre, M., Zucker-Scharff, I., Gresack, J., Allis, C.D. and Darnell, R.B.
*Cell, **170**, 1209-1223 (2017)*

Fragile X syndrome (FXS) is a leading genetic cause of intellectual disability and autism. FXS results from the loss of function of fragile X mental retardation protein (FMRP), which represses translation of target transcripts. Most of the well-characterized target transcripts of FMRP are synaptic proteins, yet targeting these proteins has not provided effective treatments. We examined a group of FMRP targets that encode transcriptional regulators, particularly chromatin-associated proteins. Loss of FMRP in mice results in widespread changes in chromatin regulation and aberrant gene expression. To determine if targeting epigenetic factors could reverse phenotypes associated with the disorder, we focused on Brd4, a BET protein and chromatin reader targeted by FMRP. Inhibition of Brd4 function alleviated many of the phenotypes associated with FXS. We conclude that loss of FMRP results in significant epigenetic misregulation and that targeting transcription via epigenetic regulators like Brd4 may provide new treatments for FXS.

3.2799 PI3K-C2α knockdown decreases autophagy and maturation of endocytic vesicles
Merill, N., Schipper, J.L., Karnes, J.B., Kauffman, A.L., Martin, K.R. and macKeigan, J.P.
*PloS One, **12(9)**, e0184909 (2017)*

Phosphoinositide 3-kinase (PI3K) family members are involved in diverse cellular fates including cell growth, proliferation, and survival. While many molecular details are known about the Class I and III PI3Ks, less is known about the Class II PI3Ks. To explore the function of all eight PI3K isoforms in autophagy, we knock down each gene individually and measure autophagy. We find a significant decrease in autophagy following siRNA-mediated *PIK3C2A* (encoding the Class 2 PI3K, PI3K-C2α) knockdown. This defective autophagy is rescued by exogenous PI3K-C2α, but not kinase-dead PI3K-C2α. Using confocal microscopy, we probe for markers of endocytosis and autophagy, revealing that PI3K-C2α colocalizes with markers of endocytosis. Though endocytic uptake is intact, as demonstrated by transferrin labeling, *PIK3C2A* knockdown results in vesicle accumulation at the recycling endosome. We isolate distinct membrane sources and observe that PI3K-C2α interacts with markers of endocytosis and autophagy, notably ATG9. Knockdown of either *PIK3C2A* or *ATG9A/B*, but not *PI3KC3*, results in an accumulation of transferrin-positive clathrin coated vesicles and RAB11-positive vesicles at the recycling endosome. Taken together, these results support a role for PI3K-C2α in the proper maturation of endosomes, and suggest that PI3K-C2α may be a critical node connecting the endocytic and autophagic pathways.

3.2800 Bacterial outer membrane vesicles suppress tumor by interferon-γ-mediated antitumor response
Kim, O.Y., Park, H.T., Dinh, N.T.H., Choi, S.J., Lee, J., Kim, J.H., Lee, S-W. and Gho, Y.S.
*Nature Communications, **8**:626 (2017)*

Gram-negative bacteria actively secrete outer membrane vesicles, spherical nano-meter-sized proteolipids enriched with outer membrane proteins, to the surroundings. Outer membrane vesicles have gained wide interests as non-living complex vaccines or delivery vehicles. However, no study has used outer membrane vesicles in treating cancer thus far. Here we investigate the potential of bacterial outer membrane vesicles as therapeutic agents to treat cancer via immunotherapy. Our results show remarkable capability of bacterial outer membrane vesicles to effectively induce long-term antitumor immune responses that can fully eradicate established tumors without notable adverse effects. Moreover, systematically administered bacterial outer membrane vesicles specifically target and accumulate in the tumor tissue, and subsequently induce the production of antitumor cytokines CXCL10 and interferon-γ. This antitumor effect is interferon-γ dependent, as interferon-γ-deficient mice could not induce such outer membrane vesicle-mediated immune response. Together, our results herein demonstrate the potential of bacterial outer

membrane vesicles as effective immunotherapeutic agent that can treat various cancers without apparent adverse effects.

3.2801 New views on phototransduction from atomic force microscopy and single molecule force spectroscopy on native rods

Maity, S., Ilieva, N., Laio, A., Torre, V. and Mazzolini, M.
*Scientific Report, **7**:12000 (2017)*

By combining atomic force microscopy (AFM) imaging and single-molecule force spectroscopy (SMFS), we analyzed membrane proteins of the rod outer segments (OS). With this combined approach we were able to study the membrane proteins in their natural environment. In the plasma membrane we identified native cyclic nucleotide-gated (CNG) channels which are organized in single file strings. We also identified rhodopsin located both in the discs and in the plasma membrane. SMFS reveals strikingly different mechanical properties of rhodopsin unfolding in the two environments. Molecular dynamic simulations suggest that this difference is likely to be related to the higher hydrophobicity of the plasma membrane, due to the higher cholesterol concentration. This increases rhodopsin mechanical stability lowering the rate of transition towards its active form, hindering, in this manner, phototransduction.

3.2802 Confounding factors in extracellular vesicle ultrafiltration and protein analysis

Vergauwen, G., Dhondt, B., Van Deun, J., Timmerman, E., gaevert, K., Braems, G., Ven den Broecke, R., Cocquyt, V., Denys, H., De Wever, O. and Hendrix, A.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract OT7.02 (2017)*

Introduction: Identification and validation of extracellular vesicle (EV)-associated functions and biomarkers requires robust isolation and characterisation protocols. We assessed the impact of commonly implemented but modified analytical variables on EV analysis.
Methods: We compared five different centrifugal filters that are often used to reduce large volume biofluids or concentrate EVs on three sample types: plasma, urine and EV-spiked PBS. Protein and nanoparticle tracking analysis was performed on the concentrate, membrane and flow through to determine EV recovery. Next, we compared three colorimetric and three fluorometric protein assay kits for their efficiency in measuring protein concentration of EV samples. In all protein assay kits the same sample volume of 5 μL EVs (1 × 1010 particles) was used. The presence and influence of OptiprepTM remnants in EV samples was assessed by DC protein assay kit-based interference of OptiprepTM at 750 nm and Q-Exactive protein analysis respectively.
Results: Regenerated cellulose with 10k pore size generated highestparticle and protein recovery of EV-spiked PBS. Other centrifugal membranes did not efficiently recover EVs with 80% reduction in particle concentration and protein concentration measurements below detection threshold due to aspecific adherence of EVs to the centrifugal membranes. Similar findings were observed for plasma and urine, however the differences were less pronounced, probably due to abundant proteins masking centrifugal filter membranes. The Qubit® protein assay kit obtained a respectively 1.5-fold and 2-fold higher protein concentration measurement with the least variance as compared to microBCA and Bradford. The OptiprepTM concentration of EV samples obtained by pelleting density fractions was estimated 1.5–2.5%, whereas no OptiprepTM remnants were detected after EV retrieval from density fractions by size-exclusion chromatography. In addition, removal of OptiprepTM remnants from EV samples improved protein identification by 40-fold as measured by number of unique proteins identified.
Conclusion: The choice of centrifugal filters and protein assay kits as well as residuals of EV isolation media can confound EV analysis and should be carefully considered when performing omics approaches and functional assays.

3.2803 Exosomes from bovine milk reduce the tumour burden and attenuates cancer cachexia

Samuel, M., Jois, M. and Mathivanan, S.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract OT9.02 (2017)*

Introduction: Milk has long been associated with good health and is one of the most consumed beverages throughout the world. Exosomes are 30–150 nm membranous vesicles of endocytic origin that are released by all cell types and are also detected in bodily fluids including milk. Whether these milk-derived exosomes can serve as cross-species messengers and have a biological effect on host organism has been poorly understood. Here, we examined the stability of bovine milk exosomes in degrading conditions and studied their biodistribution using mouse models and IVIS imaging after oral administration. We also unravel the role of bovine milk derived exosomes in colon cancer progression.

Methods: Milk exosomes were isolated using differential centrifugation and OptiPrepTM density gradient centrifugation. They were further characterised and examined for stability under harsh conditions using western blotting and nanoparticle tracking analysis. IVIS imaging system was used to study th biodistribution of these exosomes on oral gavaging. Mice models were used to understand the role of milk exosomes in cancer progression.
Result: On examining the stability of bovine milk exosomes in harsh conditions, it was concluded that these exosomes are remarkably stable in both acidic and high temperature conditions while colorectal cancer cell-derived exosomes were not. Next, we studied the biodistribution of bovine milk exosomes which suggested that orally administered milk exosomes can survive the harsh intestinal environment and can be trafficked to various organs. Interestingly, after 24 h, the milk-derived exosomes reached multiple organs including liver and spleen in the mice. To understand the role of milk-exosomes in cancer progression, in vivo mouse models implanted with colorectal cancer were orally administered with milk-derived exosomes. Remarkably, exosomes isolated from both raw and commercial (grocery store) milk significantly reduced the tumour burden. Furthermore, orally administered milk exosomes prolonged the survival of the mice by inhibition of tumourinduced weight loss in cancer cachexia mice models.
Summary: Thus this study provides new insights on the significance of milk exosomes in context of mammalian physiology as well as prompt their use as drug delivery vehicles in therapeutic interventions.

3.2804 Hepatocyte-derived exosome enrichment and cell culture methods optimisation for the identification of novel DILI biomarkers

Thacker, S., Nautiyal, M., Holman, N., Otieno, M., Watkins, P. and Mosedale, M.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract PT06.06 (2017)*

Introduction:We have previously demonstrated that hepatotoxicants induce alterations in hepatocyte-derived exosomes (HDE) prior to overt necrosis, supporting a role for HDE in the pathogenesis of drug-induced liver injury (DILI). Because HDE contain liver-specific mRNAs, miRNAs, and proteins, they may have value as sensitive and specific biomarkers of DILI. In order to explore the DILI biomarker potential of HDE, the objectives of this study were to (1) identify the best method for enrichment and (2) optimise cell culture methods to compare the number and content of HDE released from primary human hepatocytes (PHH) in response to DILI compounds.
Methods: To evaluate exosome enrichment, vesicles were isolated from the culture medium of HepG2 cells using ultracentrifugation (UC), OptiPrep density gradient ultracentrifugation (ODG), and ExoQuick-TC™ (EQ). To evaluate the effect of a Matrigel® overlay on exosome release, exosomes were enriched from the culture medium of HepaRG cells using UC. Nanoparticle tracking analysis was performed to assess vesicle number and size. Total RNA extracted from vesicles was used to determine the quantity (Quant-iT™ RiboGreen®) and fraction of miRNA that was vesicular vs. AGO2 bound (immunoprecipitation). Total protein was quantified and exosomal protein enrichment was evaluated via Western blotting.
Results: EQ resulted in a significantly higher number of exosome-sized particles than UC ($p < 0.001$) or ODG ($p < 0.0001$). Particle size and variation using UC and EQ were similar (~100 ± 10 nm), however ODG enriched for particles significantly larger in size ($p < 0.05$). EQ and UC resulted in comparable levels of vesicular RNA and protein, however UC had significantly more vesicular RNA and CD63 protein when compared to EQ or ODG ($p < 0.05$). No significant differences in particle number were observed across Matrigel concentrations ranging from 0–0.25 mg/mL.
Conclusion: These data suggest that both UC and EQ enrichment result in significantly more HDE than ODG, but UC produces a purer population of HDE. Matrigel overlay does not inhibit the release ofHDE.We conclude that UC-based enrichment provides the optimal combination of HDE quantity and purity and Matrigel overlay can be used in PHH culture for the identification of novel exosome-based biomarkers for DILI.

3.2805 Proteomic analysis of mouse lung tissue-derived vesicles, a comparison of ultracentrifugation and density flotation isolation

Lässer, C., Suzuki, S., park, K-S., Shelke, G., Hovhannisyan, L., Crescitelli, R. and Lötvall, J.
*J. Extracellular Vesicles, **6**, Suppli. A, abstract PT07.08 (2017)*

Introduction: Analysis of the proteome of extracellular vesicles (EVs) is of great importance both to identify biomarkers of disease but also to understand cell-to-cell communication in diseased tissue. The aim of this study was to establish an isolation method that isolates lung vesicles of high purity for proteomic analysis.

Methods: A mouse model for allergic asthma was used by sensitization and challenge of C57BL/6 mice to ovalbumin (OVA). Animals were sacrificed and lungs were removed and chopped in to smaller pieces that were incubated in medium for 30 minutes at 37°C and 5% CO2. Vesicles were isolated from medium either by a differential ultracentrifugation protocol (UCF) or by an Optiprep density gradient protocol (OD). Isolated vesicles were evaluated by electron microscopy (EM) and the proteome was analysed with mass spectrometry (LC-MS/MS).
Results: EM showed that both protocols isolated vesicles that where on average 40–200 nm in size. LC-MS/MS identified 1223 and 1383 proteins in the UCF and OD vesicles, respectively. Out of these, 989 proteins were detected in both samples and 88 of the top 100 exosomal proteins from the database EVpedia was identified here. Using GO Term finder it was shown that the 989 common proteins were most significantly associated with the cellular component, “extracellular exosome”, “focal adhesion” and “membrane”. The 398 uniquely identified proteins in the OD vesicles were associated with “extracellular exosome” and “membrane”, while the 234 uniquely identified proteins in the UCF vesicles were associated with “proteasome complex” and “cytoplasm”.
Conclusion: This study shows that EVs can be isolated directly from lung tissue, and these vesicles contain previously identified EV proteins. Both protocols can be used for the isolation of tissue-derived vesicles. However, flotation removes a number of contaminant proteins, including those related to the proteasome and furthermore it enriches for protein associated with membrane.

3.2806 The impact of oncogenic EGFRvIII on the proteome of extracellular vesicles released from glioblastoma cells

Choi, D-S., Montermini, L. and Rak, J.

*J. Extracellular Vesicles, **6**, Suppl. 1, abstract PT07.10 (2017)*

Glioblastoma multiforme (GBM) is the most common, highly invasive, and aggressive astrocytic brain tumour associated with poor prognosis. EGFR is amplified in a subset of GBMs and influences the invasion and proliferation of tumour cells. EGFR amplification is also often accompanied by gene rearrangements leading to the expression of constitutively active oncogenic mutant, EGFR variant III (EGFRvIII). In addition to intrinsic transformation of GBM cells themselves, EGFRvIII may also act in a non-cell-autonomous manner by virtue of intercellular trafficking of this receptor between cellular populations as cargo of extracellular vesicles (EVs). Notably, EGFRvIII may also influence EV biogenesis and alters the expression of multiple genes, but links between these events are poorly understood. To better understand how EGFRvIII contributes to tumour aggressiveness mediated by EVs, we investigated the effect of this oncogene on the EV protein composition. Thus, we employed the quantitative proteomics to analyse EVs derived from indolent parental U373 glioma cells and their EGFRvIII-expressing isogenic counterparts (U373vIII). EVs were purified using Optiprep density gradient ultracentrifugation and analysed with an UHPLC-Orbitrap Fusion Tribrid mass spectrometer. Compilation of three experimental replicates revealed remarkable changes in the expression profiles of the EV proteins, as well as changes in the release rate and concentrations of secreted EVs. For example, U373vIII-derived EVs exhibited a distinct profile of integrin expression, including elevated content of integrin α6β4, known to direct EVs to the lung. In contrast, parental U373 derived EVs carried integrin αVβ5, known to direct EVs to the liver. Thus, while GBMs generally do not metastasise to these respective organs their EVs may home to these sites and contribute, in an oncogene-specific manner, to systemic pathologies associated with brain tumours (inflammation, thrombosis). Moreover, U373vIII cells secreted EVs contained high levels of other invasion-promoting proteins including CD44, CD151, BSG. In conclusion, our results suggest that oncogenic EGFRvIII profoundly impacts the proteome of EVs released by GBM cells, and may define their biological activities beyond the content of EGFRvIII oncoprotein itself.

3.2807 Chloride intracellular channel protein 4 (CLIC4) is a serological cancer biomarker released from tumour epithelial cells via extracellular vesicles

Sanchez, V.C., Craig-Lucas, A., Wei, B-R., Shukla, A., Read, A., Lou, J., Simpson, M., Hunter, K. and Yuspa, S.

*J. Extracellular Vesicles, **6**, Suppl.1, abstract PF01.12 (2017)*

CLIC4 is a highly conserved metamorphic protein originally described as an ion channel. It translocates to the nucleus serving as an integral component of TGF-β signalling. In multiple cancers, CLIC4 is a tumour suppressor, excluded from the nucleus and lost from the cytoplasm of progressing cancer cells. In contrast, CLIC4 is upregulated in the tumour stroma in response to TGF-β. CLIC4 lacks a secretory sequence, but recent reports indicate that CLIC4 is detected in the circulation of cancer patients serving a possible

biomarker and has been detected in extracellular vesicles (EVs). EVs from cell culture supernatants or biological fluids from SKOV3/ SCID xenograft ovarian and 6DT1 orthograft breast cancer models, were isolated by differential centrifugation, following ultracentrifugation and Optiprep density gradients. EV size distribution and concentration were analysed by NTA and TEM. The presence of markers and CLIC4 were analysed by immunoblot. We validated the presence of CLIC4 in EVs released into supernatants from primary normal and multiple ovarian tumour cell lines. Substantial increases in CLIC4 were measured in EVs of tumour cells when compared to normal cells. TGF-β-induced myofibroblasts also increased CLIC4 in both the cells and the EVs they released. Immunostaining analysis of human ovarian cancer tissue arrays show CLIC4 preferentially expressed in tumour stroma of multiple subtypes with the exception of ovarian serous adenocarcinomas, where it is upregulated in both compartments. In vivo, CLIC4 levels increased in EVs released into the peritoneal cavity as tumour burden increased in a heterotopic xenograft ovarian cancer model. Moreover, CLIC4 levels in EVs isolated from plasma increased with tumour burden and lung metastatic load in an orthotopic syngeneic mouse breast cancer model. To dissect the contribution of stromal vs. tumour epithelial compartments as the source of the EVs, CLIC4 was deleted in breast cancer cell lines by CRISPR/Cas9. CLIC4 in circulating EVs is reduced in CLIC4 KO tumour-bearing mice when compared to WT, indicating that the major contribution of CLIC4 into circulation is from tumour epithelium. CLIC4 levels in EVs from biological fluids may have value as a cancer biomarker, in conjunction with other markers, to detect or analyse tumour progression or recurrence.

3.2808 Galectin-3 binding protein present at the surface of tumour exosomes contributes to their capture by stromal cells

Nakata, R., Sarte, L., Zimmermann, P. and DeClerck, Y.A.

*J. Extracellular Vesicles, **6**, Auppl. 1, abstract PH04.13 (2017)*

Introduction: Galectin-3 binding protein (Gal-3BP/LGALS3BP aka: MAC2-binding protein) is a 90 kDa secreted sialoglycoprotein that is commonly present in the cargo of exosomes and is among the 25 common cancer proteins associated with extracellular vesicles (EVs) secretion in all NCI-60 cancer cell lines (1). Here we have examined its presence and function in exosomes from human neuroblastoma cells that we had previously reported to secrete Gal-3BP (2).
Methods: The expression of Gal-3BP was examined in exosomes from 10 human NB cell lines by western blot analysis. Exosomes were prepared by differential ultracentrifugation (DUC), Optiprep density gradient centrifugation (ODGC) and size exclusion chromatography (SEC). Gal-3BP localisation in cells and exosomes was performed by confocal microscopy, flow cytometry and electron microscopy. Its role in exosome biogenesis and capture by stromal cells was examined in NB cells in which the LGAL3SBP gene was removed by CRISPR-Cas9 knock out.
Results: Gal-3BP was consistently present in all preparations of exosomes obtained from 10 NB cell lines. It was also present in exosomes from the plasma of patients with NB. It was consistently associated with exosome protein markers like CD-63, syntenin and ALIX in exosomes obtained by DUC, ODGC and SEC, in addition to being present in a soluble form in the culture medium of NB cells. However in NB cells Gal-3BP was clearly segregated from CD-63, suggesting its absence in mulitivesicular bodies and an absence of involvement in exosome biogenesis. This was further supported by the demonstration that syntenin knock down in NB cells did not affect the presence of Gal-3BP in exosomes. We then demonstrated by a combination of flow cytometry and enzymatic digestion, that Gal-3BP is present on the surface of exosomes. To better understand its function, LGALS3BP was knocked out in NB cells. Whereas Gal-3BP KO did not affect the production of exosomes in NB cells, it inhibited their capture by stroma cells.
Conclusion: Our data bring insight into the function of a protein commonly identified in the cargo of cancer cell exosomes, suggesting an absence of involvement in exosome biogenesis and a role in exosome uptake by stromal cells.
References
1. Hurwitz et al., Oncotarget 2016; 7: 86999–87015.
2. Silverman et al., Cancer Res. 2012; 72: 2228–2238.

3.2809 Histone flow: from nucleus to extracellular vesicles

Nair, R.R., Mazza, D., Agresti, A. and Bianchi, M.

*J. Extracellular Vesicles, **6**, Suppl.1, abstract OS24.04 (2017)*

Introduction: Histones play a central role in DNA packaging and epigenetic regulation. Interestingly, histones are also found as soluble molecules in the blood of sepsis patients (1). Until recently researchers

viewedhistone content in each and every cell as fixed. Recent reports indicate that histone content decreases in senescent cells. Our group had shown that macrophages treated with LPS decrease their nucleosome content by approximately 20% in 4 h (2). Our aim was to determine the fate of the 20% "missing" histones in macrophages stimulated with LPS.
Methods and Results: First, we evaluated whether stimulated macrophages reorganise their chromatin structure, at a global level. Using quantitative super-resolution microscopy (STORM) we observed that after LPS stimulation of macrophages, nucleosomes clutches (2) reduce both their size and density, suggesting that histones are evicted from chromatin. Evicted histones can have two possible fates: they can be degraded or secreted out of the cells. To test for histone degradation we collected the cells together with their medium, but we found no difference before and after stimulation. In contrast, histones amount in the medium increased after stimulation. These data imply that histones are not degraded but secreted.
The medium of stimulated macrophages was subjected to ultracentrifugation on an Optiprep density gradient. We found more histones both in extracellular vesicles (EVs) and in the soluble fraction. This result was confirmed using knock-in mice expressing H2B-GFP macrophages which were found to secrete microvesicles containing H2B-GFP. We excluded that EVs originate from membrane blebbing occurring during apoptosis and necrosis, since there is no significant apoptosis or necrosis in LPS-stimulated macrophages. However, we observed a high level of H3K4 trimethylation in the secreted histones, suggesting that they originate from the nucleus. We next investigated the localisation of histones in microvesicles: inside or outside the membrane. Biochemical experiments and STROM images indicate that histones are mostly on the outer surface of the vesicles.
Conclusion: Our data show that the nuclear histones can be evicted out of chromatin and be expelled either as soluble protein or microvesicle–associated proteins.
References
1. Chen R et al., Cell Death Dis. 2014; 5: e1370.
2. De Toma I et al., J Intern Med. 2014; 276: 454–469.

3.2810 Amoeboid cancer cells shed extracellular vesicles enriched with nuclear derived material

Sobreiro, M.R., Chen, J-F., Morley, S., You, S., Steadman, K., Gill, N.K., Chu, G.C-Y., Chung, L.W.K., Tanaka, H., Yang, W., Rowat, A.C., Tseng, H-R., Posadas, E.M., Di Vizio, D. and Freeman, M.R.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract OPT01.01 =PT01.04 (2017)*

Introduction: Deformation of the nucleus is required for migrating cells to pass through interstitial tissue spaces. However, it remains unexplored how cells modify nuclear stiffness during metastasis. Cancer cells exhibiting an "amoeboid" phenotype migrate in a manner that resembles neutrophil movement, in which nuclear deformation plays a critical role. Amoeboid tumour cells are characterised by their plasticity, ability to rapidly move through extracellular matrixes and high rates of shedding of extracellular vesicles (EVs).
Methods: Mass spectrometry, flow cytometry, differential centrifugation, iodixanol gradient, confocal 3D imaging, time lapse video microscopy, western blot, NanoVelcro Chip.
Results: Here we demonstrate that stable disruption of nuclear structure by silencing DIAPH3, emerin, or lamin A/C promotes conversion to the highly metastatic amoeboid phenotype in prostate and breast cancer cells. These amoeboid cells produced vesicles from nuclear blebs, underwent shedding of non-apoptotic EVs containing DNA, and exhibited increased sensitivity to inhibitors of DNA damage repair. Amoeboid features were detected in high grade prostate cancer, and capture of circulating tumour cells in mice and patients with metastatic prostate cancer.
Conclusion: These findings suggest the potential of incorporating the use of biomarkers of amoeboid tumour cells into clinical strategies for precision medicine.

3.2811 Attempts to re-define cellular components specifically incorporated in HIV as compared to sEVs and exosomes secreted by infected cells

Martin-Jaular, L., Liao, Z., Gerber, P.P., Ostrowski, M., Witwer, K. and Thery, C.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract OF18.02 (2017)*

Introduction: HIV, exosomes and/or other small extracellular vesicles (sEVs) share biogenesis aspects and physicochemical characteristics, making their separation difficult. Some cellular proteins are described as excluded from virions (e.g. CD45), whereas others are incorporated (e.g. CD63). We re-evaluated these results in light of our recent demonstration that many subtypes of sEVs are co-isolated by a protocol of EV isolation similar to that used for HIV isolation, and of our recently published sets of protein combinations distinguishing exosomal and non-exosomal sEVs (1). Our goal is to obtain HIV-free sEVs to allow assessing their functional properties.

Methods: Medium of Jurkat cells infected or not with VSV-G–pseudotyped NL4-3-IRES-EGFP was subjected to differential centrifugation, and velocity top-to-bottom iodixanol gradient was used to separate sEVs from virus in the 100,000g pellet (100 K). Gradient fractions were analysed by WB for the presence of different markers and by AChE assay.
Results: Differential centrifugation showed that CD45 is more abundant in large/medium EVs than in sEVs from both uninfected and infected cells. Velocity gradients revealed at least two types of sEVs in the 100 K pellet. Fractions from the top of the tube contained CD9 and some CD45 but little or no CD63 (i.e. non-exosomal sEVs), whereas intermediate fractions contained CD9, CD63, and syntenin-1, hence probably exosomes. Gag and CD63 but little or no CD9, Syntenin-1 and CD45 were detected in bottom fractions of infected cells' 100 K pellet. Importantly, AChE activity was found in fractions different from those enriched in Gag but also from those enriched for the other sEVs/exosome markers.
Conclusions: Despite exclusion from virus containing fractions, neither AChE activity nor CD45 are satisfying markers to distinguish HIV from exosomes. Velocity gradients achieve some separation of sEVs/exosome or virus markers, but overlap of distribution makes it difficult to use them for unbiased proteomic comparisons. Further work will be required to identify, if they exist, sEV and/or exosomal components specifically excluded from HIV virions.
Reference
1. Kowal et al., PNAS 2016; 113: E968.

3.2812 Characterisation of extracellular vesicles with milk fat globule membrane-like properties that carry most microRNAs in commercial dairy cow milk

Abderrahim, B., Sophia, L., Ting, S.S., Laugier, J., Boilard, E., Caroline, G. and Provost, P.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract OS21.02 (2017)*

Introduction: MicroRNAs are short (~22 nucleotides), non-coding RNAs that play an essential role in post-transcriptional gene regulation. Found in several biological fluids, including milk, they are often associated with extracellular vesicles (EVs), like exosomes. In a previous study, we found that commercial dairy cow milk microRNAs resist digestion in vitro. Surprisingly, we observed thatmost of them sediment at low centrifugation speed, thereby challenging their association with exosomes in commercial milk.
Methods: We used differential ultracentrifugation and iodixanol density gradient (IDG) to isolate milk EVs, which we analysed for microRNA enrichment by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) and for EV-associated proteins by western blot. We further characterised these EVs by density measurements, fluorescence RNA labelling, mass spectrometry (LC-MS/MS), dynamic light scattering (DLS), flow cytometry, transmission electron microscopy (TEM) and proteinase K assay.
Results: We found no correlation between bta-miR-223 and bta-miR-125b and exosome-associated proteins found in low speed ultracentrifugation pellets (i.e. 12,000g and 35,000g), but a positive correlation ($p < 0.05$) between bta-miR-125b and xanthine dehydrogenase (XDH). Two IDG fractions were highly enriched in double stranded RNAs and microRNAs, contained several exosome-associated proteins and most of the exosomelike EVs found in these gradients. However, proteinase K assay and subsequent LC-MS/MS analysis challenged the exosome nature of these EVs, as all exosome-enriched proteins were digested during the assay and these digested EVs were found to contain milk fat globule membrane (MFGM)-enriched proteins, including immunomodulatory XDH, butyrophilin 1A1 (BTN1A1), mucin (MUC-1) and lactadherin (MFG-E8).
Conclusion: Our results suggest the presence of exosome-like EVs with MFGM-like properties in commercial milk and their association with the majority of milk microRNAs. Considering their resistance to proteinase K digestion and bioaccessibility in vitro, these EVs may contribute to interspecies transfer of dietary microRNAs and immune regulation by milk EVs, which require further investigations.

3.2813 Recipient cell organelle separation for EV uptake studies: Tracking of extracellular vesicles

Shelke, G. and Lötvall, J.
*J. Extracellular Vesicles, **6**, Suppl. 1, abstract LBP.45 (2017)*

Background: Extracellular vesicles (EVs) such as exosomes and microvesicle are known to delivery cargo like proteins, lipids, RNA, and DNA to the recipient cells. Transfer of EVs to recipient cells to deliver these cargos is essential to induce cellular phenotypic changes. Current methods to localize EVs in recipient cells are restricted to imaging of cells using co-localization of fluorescent probes. We propose a physical method that provides high-resolution separation of organelles that can be associated with EVs recipient cell trafficking.

Methods: EVs were isolated from mast cell line (HMC1.2) by differential centrifugation (16,500´g 20 min and 120,000´g 3 hr) followed by flotation on iodixanol gradient (182,300´g for 16 hours; SW40-Ti rotor). EVs were biotinylated by incubating it with EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) and free biotin was removed by dialysis (3.5 kDa filter) as per the manufacturer recommendations. Biotinylated-EVs were later incubated with HEK-293T cells for 60 min, after which cells were lysed (High salt, high pH buffer and sonication) to obtain crude organelles. Crude organelles carrying biotinylated EVs were further separated on iodixanol density gradient with two consecutive ultracentrifugation steps. Various iodixanol fractions were analyzed using immunoblotting for lysosomal (LAMP1) and endosomal protein (EEA1), as well as streptavidin-HRP based detection of EVs-biotin.
Results: High resolution separation of endosomal and lysosomal organelles fraction was obtained using this method. We found that biotinylated EV proteins were enriched in the endosomal fraction. A small quantity of biotinylated-EV proteins were also present in lysosomal enriched fraction.
Summary/Conclusion: Endosomal and lysosomal localization of EVs can be performed in recipient cell by iodixanol density gradient centrifugation. EVs were primarily enriched in the endosomal compartment, and only traces were detected in the endo-lysosomal compartment at the time point studied.

3.2814 Isolation of Lipid Raft Proteins from CD133+ Cancer Stem Cells
Gupta, V.K. and Banerjee, S.
*Methods in Mol. Biol., **1609**, 25-31 (2017)*

Pancreatic cancer cells expressing the surface markers CD133 have been widely reported as cancer stem cells and mainly responsible for tumor recurrence and chemoresistance in pancreatic cancer. In spite of its role as a stem cell marker in pancreatic cancer, its function remains elusive. CD133 (also known as prominin-1) is a pentaspan glycoprotein predominantly localized in lipid rafts, specialized membrane microdomains enriched in crucial signaling proteins. Coexistence of CD133 with these signaling proteins can modulate various signaling pathways that might be responsible for aggressive phenotype of CD133+ cells. This chapter describes a detailed protocol to isolate lipid rafts from CD133+ tumor initiating cells. Purified lipid rafts can be investigated further for protein or lipid composition by mass spectrometry that can shed some light on functional role of CD133 protein in these cancer stem cells.

3.2815 Analysis of Ras/ERK Compartmentalization by Subcellular Fractionation
Agudo-Ibanez, L., Crespo, P and Casar, B.
*Methods in Mol. Biol., **1487**, 151-162 (2017)*

A vast number of stimuli use the Ras/Raf/MEK/ERK signaling cascade to transmit signals from their cognate receptors, in order to regulate multiple cellular functions, including key processes such as proliferation, cell cycle progression, differentiation, and survival. The duration, intensity and specificity of the responses are, in part, controlled by the compartmentalization/subcellular localization of the signaling intermediaries. Ras proteins are found in different plasma membrane microdomains and endomembranes. At these localizations, Ras is subject to site-specific regulatory mechanisms, distinctively engaging effector pathways and switching-on diverse genetic programs to generate a multitude of biological responses. The Ras effector pathway leading to ERKs activation is also subject to space-related regulatory processes. About half of ERK1/2 substrates are found in the nucleus and function mainly as transcription factors. The other half resides in the cytosol and other cellular organelles. Such subcellular distribution enhances the complexity of the Ras/ERK cascade and constitutes an essential mechanism to endow variability to its signals, which enables their participation in the regulation of a broad variety of functions. Thus, analyzing the subcellular compartmentalization of the members of the Ras/ERK cascade constitutes an important factor to be taken into account when studying specific biological responses evoked by Ras/ERK signals. Herein, we describe methods for such purpose.

3.2816 Extracellular vesicles from mesenchymal stem cells activates VEGF receptors and accelerates recovery of hindlimb ischemiaGangadaran, P., Rajendran, R.L., Lee, H.W., kalimuthu, S., Hong, C.M., Jeong, S.Y., Lee, S-W., Lee, J. and Ahn, B-C.
J. Controlled Release, 264, 112-126 (2017)

Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) are potential therapies for various diseases, but their angiogenic mechanisms of therapeutic efficacy remain unclear. Here, we describe how MSC-EVs, activates VEGF receptors and downstream angiogenesis pathways. Mouse MSC-EVs were isolated from cell culture medium and characterized using transmission electron microscopy, nanoparticle analysis, and western blotting. In vitro migration, proliferation, and tube formation assays

using endothelial cells were used to assess the angiogenic potential of MSC-EVs, and revealed higher levels of cellular migration, proliferation, and tube formation after treatment. qRT-PCR and western blotting (WB) revealed higher protein and mRNA expression of the angiogenic genes VEGFR1 and VEGFR2 in mouse SVEC-4 endothelial cells after MSC-EVs treatment. Additionally, other vital pro-angiogenic pathways (SRC, AKT, and ERK) were activated by in vitro MSC-EV treatment. WB and qRT-PCR revealed enriched presence of VEGF protein and miR-210-3p in MSC-EV. The hindlimb ischemia mouse model was established and MSC-EVs with or without Matrigel (EV-MSC + Gel) were injected into the ischemic area and blood reperfusion was monitored using molecular imaging techniques. The in vivo administration of MSC-EVs increased both blood reperfusion and the formation of new blood vessels in the ischemic limb, with the addition of matrigel enhancing this effect further by releasing EVs slowly. MSC-EVs enhance angiogenesis in ischemic limbs, most likely via the overexpression of VEGFR1 and VEGFR2 in endothelial cells. These findings reveal a novel mechanism of activating receptors by MSC-EVs influence the angiogenesis.

3.2817 Fractionation Techniques to Examine Effector Translocation
Olson, R.M. and Anderson, D.M.
Methods in Mol. Biol., ***1531***, *101-109 (2017)*

Many Gram-negative bacterial pathogens use type III secretion systems to export proteins that act directly on the host and aid in the infectious process. Extracellular bacteria primarily rely upon the type III secretion system to insert or inject effector proteins into the cytosol of their host cell in order to perturb intracellular signaling events and aid in pathogenesis. Intracellular bacteria can also depend on the T3SS translocation of effector proteins from vacuolar compartments into the vacuolar membrane or host cell cytosol where they can modulate intracellular trafficking and/or signaling pathways necessary for their growth and survival. Biochemical fractionation of infected cells in vitro enables detection of these events, making it possible to identify relevant protein–protein interactions, characterize phenotypes of mutant strains and understand how these effector proteins impact host cells. In this chapter we provide methods for the analysis of translocated effector proteins using biochemical and mechanical fractionation procedures.

3.2818 Analysis of Mitochondrial Membrane Protein Complexes by Electron Cryo-tomography
Gold, V.A.M., Brandt, T., Cavellini, L., Cohen, M.M., Leva, R. and van der Laan, M.
Methods in Mol. Biol., ***1567***, *315-336 (2017)*

The visualization of membrane protein complexes in their natural membrane environment is a major goal in an emerging area of research termed structural cell biology. Such approaches provide important information on the spatial distribution of protein complexes in their resident cellular membrane systems and on the structural organization of multi-subunit membrane protein assemblies. We have developed a method to specifically label active membrane protein complexes in their native membrane environment with electron-dense nanoparticles coupled to an activating ligand, in order to visualize them by electron cryo-tomography. As an example, we describe here the depiction of preprotein import sites of mitochondria, formed by the translocase of the outer membrane (TOM complex) and the presequence translocase of the inner membrane (TIM23 complex). Active import sites are selectively labeled via a biotinylated, quantum dot-coupled preprotein that is arrested in translocation across the outer and inner mitochondrial membranes. Additionally, a related method is described for direct labeling of mitochondrial outer membrane proteins that does not depend on binding of a ligand.

3.2819 Analysis of Fatty Acid and Cholesterol Content from Detergent-Resistant and Detergent-Free Membrane Microdomains
McClellan, M.E. and Eliiott, M.H.
Methods in Mol. Biol., ***1609***, *185-194 (2017)*

The compartmentalization of cellular membranes into discrete membrane microdomains (known as *lipid rafts*) challenged the original definition of membranes as containing randomly distributed lipid and protein components. The lipid microdomain hypothesis has generated significant controversy and rigorous inquiry based on the attractive idea that such domains concentrate machinery to mediate cellular events such as signaling and endocytosis. As such, numerous studies have used biochemical, cell biological, and biophysical methodologies to define the composition of such domains in a variety of experimental contexts. In this chapter, we describe methodologies to isolate membranes from cell or tissue sources with

biophysical/biochemical properties of membrane microdomains that are amenable to subsequent classical or mass spectrometry-based lipid analytical approaches.

3.2820 Isolation of Extracellular Vesicles by Ultracentrifugation
Momen-Heravi, F.
Methods in Mol. Biol., ***1660***, *25-32 (2017)*

Extracellular vesicles (EVs) represent a group of heterogeneous vesicles that can be obtained from almost all biofluids. EVs, including microvesicles, exosomes, and apoptotic bodies, can deliver bioactive cargos and signaling molecules. Various physiological roles and pathophysiological roles for EVs in diseases such as cancer, infectious diseases, endocrine diseases, and neurodegenerative disorders have been recognized. These observations highlight EVs as potential novel biomarkers and targets for therapeutic intervention. One of the major limitations in the use of EVs for diagnosis and therapeutic purposes is the lack of standardization of isolation techniques. Here, we describe protocols for ultracentrifugation and sucrose gradient isolation methods, which are the current gold standard, and are the most studied methods for EV isolation.

3.2821 Purification Protocols for Extracellular Vesicles
Lane, R.E., Korbie, D., Trau, M. And Hill, M.M.
Methods in Mol. Biol., ***1660***, *111-130 (2017)*

This chapter provides a description of some of the standard methods used for the isolation of extracellular vesicles (EVs) from a variety of biological fluids, including cell culture media, urine, plasma and serum. The methods presented include ultracentrifugation, ultrafiltration, proprietary polymer-based reagents, size exclusion chromatography, density gradient separation, and immunoaffinity capture. Ultracentrifugation methods use high speed centrifugation to pellet vesicles, whilst polymer-based reagents are added to the sample to facilitate vesicle precipitation using lower speeds. Ultrafiltration involves the concentration of vesicles from a large volume of biological fluid using a centrifugal filter unit. Size exclusion chromatography and density gradient separation are both designed to allow the separation of vesicles from other nonvesicular debris. Immunoaffinity capture methods use antibody-coated beads to selectively isolate vesicles displaying a surface marker of interest. Ultimately, the choice of purification method for an individual experiment is influenced by time, cost, and equipment considerations, as well as the sample requirements for any downstream analyses.

3.2822 Extraction and Analysis of Extracellular Vesicle-Associated miRNAs Following Antibody-Based Extracellular Vesicle Capture from Plasma Samples
Zocco, D. and Zarovni, N.
Methods in Mol. Biol., ***1660***, *269-285 (2017)*

Extracellular vesicle (EV)-associated RNAs (EV-RNA) are under intense investigation due to their potential role in health and disease. Several approaches are currently employed to isolate blood-derived EVs for RNA analysis, most of which are either time-consuming and expensive, such as methods based on EVs physical properties (ultracentrifugation and Optiprep density gradient), or also copurify blood contaminants, mostly protein aggregates and immune complexes, (such as chemical precipitation). In addition, there is a lack of standardized protocols for the extraction of EV-RNA and very little consensus on the technological platforms and normalization tools for assessing the expression levels of different RNA species. These methodological issues complicate the comparison between independent data sets, potentially biasing results and conclusions.
In this book chapter we propose a protocol that might overcome some of the abovementioned issues through antibody-based isolation of blood-derived EVs followed by extraction and expression analysis of small-RNA species (miRNA) by reverse transcriptase quantitative PCR (RT-qPCR). The advantages of immunoaffinity approaches over other isolation methods are multiple and include: (1) the selective enrichment of specific EV subpopulations with restricted tissue/cell origin, (2) reduction of matrix effects and blood contaminants that may confound miRNA profiling from complex biological fluids and (3) easy coupling to conventional quantitative assays (e.g., RT-qPCR). In conclusion, we describe a protocol for standard enrichment and quantitative analysis of EV-miRNAs from blood and we warrant for technological improvements, such as the use of novel biomaterials, surface chemistries, binding agents and assay/sensor design that may further improve it.

3.2823 An Adaptable Polyethylene Glycol-Based Workflow for Proteomic Analysis of Extracellar Vesicles
Hurwitz, S.N. and Meckes Jr., D.G.
Methods in Mol. Biol., ***1660****, 303-317 (2017)*

Extracellular vesicles (EVs), including exosomes are endocytically derived nanovesicles expelled from cells that contain molecular information in the form of lipids, proteins, and nucleic acids. Transfer of this information to other cells in local or distant microenvironments facilitates cell-to-cell communication. Importantly, diseased cells release exosomes containing specific cargo that may contribute to pathology and can be harnessed for diagnostic or prognostic use. The broad potential medical utility of exosomes has fueled rapidly expanding research on understanding the composition and functions of exosomes in normal and pathological conditions. Here, we provide a complete workflow for purifying exosome-sized vesicles from biological fluids for in-depth proteomic analyses. Moreover, this polyethylene glycol-based method is efficient, highly adaptable, and compatible with a variety of downstream applications.

3.2824 Isolation of Extracellular Vesicles in Saliva Using Density Gradient Ultracentrifugation
Iwai, K., Yamamoto, S., Yoshida, M. and Shiba, K.
Methods in Mol. Biol., ***1660****, 343-350 (2017)*

This chapter describes a method for isolating human salivary extracellular vesicles (EVs) using density gradient ultracentrifugation. Standard protocols established for isolation of EVs from blood or a conditioned medium of cultured cells do not work for whole saliva, due to its viscosity. Therefore, procedures including a pretreatment step and utilizing iodixanol as a gradient material enable EVs to be concentrated to a 1.1 g/ml density. This protocol is compatible with both swing and angle rotors. By employing an angle rotor, which enables high g-force, the centrifugation time was reduced to 4 h from the 17 h required when using a swing rotor.

3.2825 Microcapillary Chip-Based Extracellular Vesicle Profiling System
Akagi, T. and Ichiki, T.
Methods in Mol. Biol., ***1660****, 209-217 (2017)*

A microcapillary chip-based particle electrophoresis system developed for characterizing extracellular vesicles (EVs) is described. So far, it is technologically difficult to analyze or identify a heterogeneous population of particles ranging from several tens to one hundred nanometers, and hence, there is a growing demand for a new analytical method of nanoparticles among researchers working on extracellular vesicles. The analytical platform presented in this chapter allows detection of individual nanoparticles or nanovesicles of less than 50 nm in diameter and enables the characterization of nanoparticles based on multiple indexes such as concentration, diameter, zeta potential, and surface antigenicity. This platform will provide a useful and easy-to-use solution for obtaining both quantitative and qualitative information on EV samples used in research and development of exosome biology and medicine.

3.2826 Purification of LAT-Containing Membranes from Resting and Activated T Lymphocytes
Hivroz, C., larghi, P., Jouve, M. and Ardouin, L.
Methods in Mol. Biol., ***1584****, 355-368 (2017)*

In T lymphocytes, the immune synapse is an active zone of vesicular traffic. Directional transport of vesicular receptors and signaling molecules from or to the immune synapse has been shown to play an important role in T-cell receptor (TCR) signal transduction. However, how vesicular trafficking is regulating the activation of T cells is still a burning question, and the characterization of these intracellular compartments remains the first step to understand this process. We describe herein a protocol, which combines a separation of membranes on flotation gradient with an affinity purification of *Strep*-tagged fusion transmembrane proteins with *Strep*-Tactin® resin, allowing the purification of membranes containing the *Strep*-tagged molecule of interest. By keeping the membranes intact, this protocol leads to the purification of molecules physically associated with the *Strep*-tagged protein as well as of molecules present in the same membrane compartment: transmembrane proteins, proteins strongly associated with the membranes, and luminal proteins. The example shown herein is the purification of membrane compartment prepared from T lymphocytes expressing LAT fused to a *Strep*-tag.

3.2827 **Surface Profiling of Extracellular Vesicles from Plasma or Ascites Fluid Using DotScan Antibody Microarrays**
Belov, L., Hallal, S., Matic, K., Zhou, J., Wissmueller, S., Ahmed, N., Tanjil, S., Mulligan, S.P., Best, O.G., Simpson, R.J. and Christopherson, R.I.
Methods in Mol. Biol., ***1619***, *263-301 (2017)*

DotScan antibody microarrays were initially developed for the extensive surface profiling of live leukemia and lymphoma cells. DotScan's diagnostic capability was validated with an extensive clinical trial using mononuclear cells from the blood or bone marrow of leukemia or lymphoma patients. DotScan has also been used for the profiling of surface proteins on peripheral blood mononuclear cells (PBMC) from patients with HIV, liver disease, and stable and progressive B-cell chronic lymphocytic leukemia (CLL). Fluorescence multiplexing allowed the simultaneous profiling of cancer cells and leukocytes from disaggregated colorectal and melanoma tumor biopsies after capture on DotScan. In this chapter, we have used DotScan for the surface profiling of extracellular vesicles (EV) recovered from conditioned growth medium of cancer cell lines and the blood of patients with CLL. The detection of captured EV was performed by enhanced chemiluminescence (ECL) using biotinylated antibodies that recognized antigens expressed on the surface of the EV subset of interest. DotScan was also used to profile EV from the blood of healthy individuals and the ascites fluid of ovarian cancer patients. DotScan binding patterns of EV from human plasma and other body fluids may yield diagnostic or prognostic signatures for monitoring the incidence, treatment, and progression of cancers.

3.2828 **A Protein Scaffold Coordinates SRC-Mediated JNK Activation in Response to Metabolic Stress**
Kant, S., Standen, C.L., Morel, C., Swat, W., Flavell, R.A. and Davis, R.J:
Cell Reports, ***20***, *2775-2783 (2017)*

Obesity is a major risk factor for the development of metabolic syndrome and type 2 diabetes. How obesity contributes to metabolic syndrome is unclear. Free fatty acid (FFA) activation of a non-receptor tyrosine kinase (SRC)-dependent cJun NH_2-terminal kinase (JNK) signaling pathway is implicated in this process. However, the mechanism that mediates SRC-dependent JNK activation is unclear. Here, we identify a role for the scaffold protein JIP1 in SRC-dependent JNK activation. SRC phosphorylation of JIP1 creates phosphotyrosine interaction motifs that bind the SH2 domains of SRC and the guanine nucleotide exchange factor VAV. These interactions are required for SRC-induced activation of VAV and the subsequent engagement of a JIP1-tethered JNK signaling module. The JIP1 scaffold protein, therefore, plays a dual role in FFA signaling by coordinating upstream SRC functions together with downstream effector signaling by the JNK pathway.

3.2829 **Identification of syntaxin 4 as an essential factor for the hepatitis C virus life cycle**
Ren, H., Elgner, F., Himmelsbach, K., Akhras, S., Jiang, B., Medvedev, R., Ploen, D. and Hildt, E.
Eur. J. Cell Biol., ***96***, *542-552 (2017)*

Although there is evidence that multivesicular bodies (MVBs) are involved in the release of hepatitis C virus (HCV), many aspects of HCV release are still not fully understood. The amount of α-taxilin that prevents SNARE (soluble *N*-ethylmaleimidesensitive factor attachment protein receptor) complex formation by binding to free syntaxin 4 is reduced in HCV-positive cells. Therefore, it was analyzed whether the t-SNARE protein syntaxin 4 which mediates vesicles fusion is involved in the HCV life cycle. HCV-positive cells possess an increased amount of syntaxin 4 protein, although the amount of syntaxin 4-specific transcripts is decreased in HCV-positive Huh7.5 cells and in HCV-infected primary human hepatocytes. In HCV-positive cells a significant longer half-life of syntaxin 4 was found that overcompensates for the decreased expression and leads to the elevated level of syntaxin 4. Overexpression of syntaxin 4 reduces the intracellular amount of infectious viral particles by facilitating viral release, while silencing of syntaxin 4 expression using specific siRNAs inhibits the release of HCV particles and so leads to an increase in the intracellular amount of infectious viral particles. This indicates that HCV uses a SNARE-dependent pathway for viral release. Confocal immunofluorescence microscopy revealed a colocalization of syntaxin 4 with a MVB-specific marker, exosomes and HCV core, which suggests a fraction of syntaxin 4 is associated with exosomes loaded with HCV. Altogether, it is assumed that syntaxin 4 is a novel essential cellular factor for the release of HCV.

3.2830 **Flow Cytometric and Sorting Analyses for Nuclear DNA Content, Nucleotide Sequencing, and Interphase FISH**
Kaeser, G. and Chun, J.

*Neuromethods, **131**, 43-55 (2017)*

The study of genomic mosaicism among human brain cells is challenging. The human brain contains hundreds of billions of cells that are intricately connected and difficult to separate as intact, single cells. Additional challenges are encountered when interrogating small, seemingly random changes within single-cell genomes. Flow cytometric analysis (**FCM**), and fluorescence-activated *nuclear* sorting (**FANS**), has expanded our assessment capabilities for global and specific genomic and transcriptomic changes in human brain cells. The general approach is being utilized in a variety of downstream applications by many laboratories. Here we provide detailed methods of nuclear DNA content assessment and sorting that reports population averages as well as single-cell nuclear DNA content from cells of the human brain. We highlight protocol modifications that allow the same nuclear preparation to be used for subpopulation-specific FANS (also see chapter "Single-Cell Whole Genome Amplification and Sequencing to Study Neuronal Mosaicism and Diversity") in downstream analyses such as fluorescent in situ hybridization (**FISH**) (see chapters "FISH-Based Assays for Detecting Genomic (Chromosomal) Mosaicism in Human Brain Cells," "FISH Analysis of Aging-Associated Aneuploidy in Neurons and Non-neuronal Brain Cells" and "Using Fluorescence In Situ Hybridization (FISH) Analysis to Measure Chromosome Instability and Mosaic Aneuploidy in Neurodegenerative Diseases"), and single-cell genomic and transcriptomic sequencing (see chapters "Flow Cytometric Quantification, Isolation, and Subsequent Epigenetic Analysis of Tetraploid Neurons," "Single Cell CNV Detection in Human Neuronal Nuclei," "Multiple Annealing and Looping-Based Amplification Cycles (MALBAC) for the Analysis of DNA Copy Number Variation," and "Single-Cell Whole Genome Amplification and Sequencing to Study Neuronal Mosaicism and Diversity"). Other downstream techniques include, but are not limited to, single-cell qPCR (see chapter "Competitive PCR for Copy Number Assessment by Restricting dNTPs") and estimation of line-1 copy number (see chapters "Analysis of LINE-1 Retrotransposition in Neural Progenitor Cells and Neurons," "Estimation of LINE-1 Copy Number in the Brain Tissue and Isolated Neuronal Nuclei," and "Analysis of Somatic LINE-1 Insertions in Neurons").

3.2831 Single-Cell CNV Detection in Human Neuronal Nuclei
Wierman, M.B., Burbulis, I.E., Chronister, W.D., Bekiranov, S. and McConnell, M.J.
*Neuromethods, **131**, 109-131 (2017)*

Genomic mosaicism is prevalent throughout human somatic tissues and is much more common than previously thought. Here, we describe step-by-step methods to isolate neuronal nuclei from human brain and identify megabase-scale copy number variants (CNVs) in single nuclei. The approach detailed herein includes use of CellRaft technology for single-nucleus isolation, the PicoPLEX approach to whole-genome amplification and library preparation, and a pooled library purification protocol, termed Gel2Gel, which has been developed in our laboratory. These methods are focused toward neuroscience research, but are adaptable to many biomedical fields.

3.2832 Identification of Low Allele Frequency Mosaic Mutations in Alzheimer Disease
Frigerio, C.S., Fiers, M., Voet, T. and De Strooper, B.
*Neuromethods, **131**, 361-378 (2017)*

Germline mutations of*APP*,*PSEN1*, and*PSEN2* genes cause autosomal dominant Alzheimer disease (AD). Somatic variants of the same genes may underlie pathogenesis in sporadic AD, which is the most prevalent form of the disease. Importantly, such somatic variants may be present at very low allelic frequency, confined to the brain, and are thus very difficult or impossible to detect in blood-derived DNA. Ever-refined methodologies to identify mutations present in a fraction of the DNA of the original tissue are rapidly transforming our understanding of DNA mutation and their role in complex pathologies such as tumors. These methods stand poised to test to what extend somatic variants may play a role in AD and other neurodegenerative diseases.

3.2833 Single-Cell Whole Genome Amplification and Sequencing to Study Neuronal Mosaicism and Diversity
Reed, P.J., Wang, M., Erwin, J.A., Paquola, A.C.M. and Gage, F.H.
*Neuromethods, **131**, 253-268 (2017)*

Neuronal mosaicism describes the extent of intercellular genotypic diversity within a single human brain. This somatic variability is driven by numerous mechanisms including errors in DNA replication acquired throughout development and by the activity of endogenous retrotransposons. The study of

retrotransposition in neuronal mosaicism may prove crucial to understanding the true complexity of normal and aberrant brain function. Specifically, numerous lines of evidence suggest that retrotransposition specific aspects of neuronal mosaicism may contribute to the unresolved etiology of many neurologic and neuropsychiatric disorders. Here, we describe the SLAV-Seq method, a recent advancement in the field over previous approaches used to study the diversity of LINE-1 based neuronal mosaicism at the single-cell level. We describe in detail, methodology for the isolation of single cells from bulk tissue by FACS, the amplification of single-cell genomic DNA by multiple displacement amplification (MDA), the targeted enrichment of LINE-1 somatic events, and the sequencing of the LINE-1 enriched library. Finally, we discuss methods for the quantification and analysis of the neuronal mosaicism identified by SLAV-Seq and some of the current technical limitations.

3.2834 **Therapeutic Applications of Extracellular Vesicles: Perspectives from Newborn Medicine**
Willis, G.R., Kourembanas, S. and Mitsialis, S.A.
Methods in Mol. Biol., ***1660****, 409-432 (2017)*

With the advancements in antenatal steroid therapies and surfactant replacement, current clinical practices in neonatal intensive care units allow the survival of infants at very low gestational age. Despite these advances, there continues to be significant morbidity associated with extreme preterm birth that includes both short-term and long-term cardiorespiratory impairment. With no effective single therapy in preventing or treating developmental lung injuries, the need for new tools to treat and reduce risk of complications associated with extreme preterm birth is urgent. Stem cell-based therapies, in particular therapies utilizing mesenchymal stem (stromal) cells (MSCs), have shown promise in a number of animal models of lung pathologies relevant to neonatology. Recent studies in this field have consolidated the concept that the therapeutic mechanism of MSC action is paracrine, and this led to wide acceptance of the concept that the delivery of the MSC secretome rather than live cells may provide an alternative therapeutic approach for many complex diseases. Here, we summarize the significance and application of cell-free based therapies in preclinical models of neonatal lung injury. We emphasize the development of extracellular vesicle (EV)-based therapeutics and focus on the challenges that remain to be addressed before their application to clinical practice.

3.2835 **Designer outer membrane vesicles as immunomodulatory systems – Reprogramming bacteria for vaccine delivery**
Gnopo, Y.M.D., Watkins, H.C., Stevenson, T.C., DeLisa, M.P. and Putnam, D.
Adv. Drug Delivery Reviews, ***114****, 132-142 (2017)*

Vaccines often require adjuvants to be effective. Traditional adjuvants, like alum, activate the immune response but in an uncontrolled way. Newer adjuvants help to direct the immune response in a more coordinated fashion. Here, we review the opportunity to use the outer membrane vesicles (OMVs) of bacteria as a way to modulate the immune response toward making more effective vaccines. This review outlines the different types of OMVs that have been investigated for vaccine delivery and how they are produced. Because OMVs are derived from bacteria, they have compositions that may not be compatible with parenteral delivery in humans; therefore, we also review the strategies brought to bear to detoxify OMVs while maintaining an adjuvant profile. OMV-based vaccines can be derived from the pathogens themselves, or can be used as surrogate constructs to mimic a pathogen through the heterologous expression of specific antigens in a desired host source strain, and approaches to doing so are reviewed. Additionally, the emerging area of engineered pathogen-specific carbohydrate sequences, or glycosylated OMVs is reviewed and contrasted with protein antigen delivery. Existing OMV-based vaccines as well as their routes of administration round out the text. Overall, this is an exciting time in the OMV field as it matures and leads to more effective and targeted ways to induce desired pathogen-specific immune responses.

3.2836 **Male hormones activate EphA2 to facilitate Kaposi's sarcoma-associated herpesvirus infection: Implications for gender disparity in Kaposi's sarcoma**
Wang, X., Zou, Z., Deng, Z., Liang, D., Zhou, X., Sun, R. and Lan, K.
PloS Pathogens, ***13(9)****, e1006580 (2017)*

There is increasing consensus that males are more vulnerable than females to infection by several pathogens. However, the underlying mechanism needs further investigation. Here, it was showed that knockdown of androgen receptor (AR) expression or pre-treatment with 5α-dihydrotestosterone, the AR agonist, led to a considerably dysregulated Kaposi's sarcoma-associated herpesvirus (KSHV) infection. In

endothelial cells, membrane-localized AR promoted the endocytosis and nuclear trafficking of KSHV. The AR interacted with ephrin receptor A2 (EphA2) and increased its phosphorylation at residue Ser897, which was specifically upregulated upon KSHV infection. This phosphorylation resulted from the AR-mediated recruitment of Src, which resulted in the activation of p90 ribosomal S6 kinase 1 (RSK1), which directly phosphorylates EphA2 at Ser897. Finally, the EphA2-mediated entry of KSHV was abolished in a Ser897Asn EphA2 mutant. Taken together, membrane-localized AR was identified as a KSHV entry factor that cooperatively activates Src/RSK1/EphA2 signaling, which subsequently promotes KSHV infection of both endothelial and epithelial cells.

3.2837 **Erdj3 Has an Essential Role for Z Variant Alpha-1-Antitrypsin Degradation**
Khodayari, N., marek, G., Lu, Y., Krotova, K., Wang, R.L. and Brantly, M.
J. Cell. Biochem., ***118***, *3090-3101 (2017)*

Alpha-1-antitrypsin deficiency (AATD) is an inherited disease characterized by emphysema and liver disease. AATD is most often caused by a single amino acid substitution at amino acid 342 in the mature protein, resulting in the Z mutation of the alpha-1-antitrypsin gene (ZAAT). This substitution is associated with misfolding and accumulation of ZAAT in the endoplasmic reticulum (ER) of hepatocytes and monocytes, causing a toxic gain of function. Retained ZAAT is eliminated by ER-associated degradation and autophagy. We hypothesized that alpha-1-antitrypsin (AAT)-interacting proteins play critical roles in quality control of human AAT. Using co-immunoprecipitation, we identified ERdj3, an ER-resident Hsp40 family member, as a part of the AAT trafficking network. Depleting ERdj3 increased the rate of ZAAT degradation in hepatocytes by redirecting ZAAT to the ER calreticulin-EDEM1 pathway, followed by autophagosome formation. In the Huh7.5 cell line, ZAAT ER clearance resulted from enhancing ERdj3-mediated ZAAT degradation by silencing ERdj3 while simultaneously enhancing autophagy. In this context, ERdj3 suppression may eliminate the toxic gain of function associated with polymerization of ZAAT, thus providing a potential new therapeutic approach to the treatment of AATD-related liver disease.

3.2838 **Visualization of cytosolic ribosomes on the surface of mitochondria by electron cryo-tomography**
Gold, V.A.M., Chroscicki, P., Bragoszewski, P. and Chacinska, A.
EMBO Reports, ***18(10)***, *1786-1800 (2017)*

We employed electron cryo-tomography to visualize cytosolic ribosomes on the surface of mitochondria. Translation-arrested ribosomes reveal the clustered organization of the TOM complex, corroborating earlier reports of localized translation. Ribosomes are shown to interact specifically with the TOM complex, and nascent chain binding is crucial for ribosome recruitment and stabilization. Ribosomes are bound to the membrane in discrete clusters, often in the vicinity of the crista junctions. This interaction highlights how protein synthesis may be coupled with transport. Our work provides unique insights into the spatial organization of cytosolic ribosomes on mitochondria.

3.2839 **Fatty acid binding protein (Fabp) 5 interacts with the calnexin cytoplasmic domain at the endoplasmic reticulum**
Jung, J., Wang, J., Groenendyk, J., Lee, D., Michalak, m. and Agellon, L.B.
Biochem. Biophys. Res. Comm., ***493***, *202-206 (2017)*

Calnexin is a type 1 integral endoplasmic reticulum membrane molecular chaperone with an endoplasmic reticulum luminal chaperone domain and a highly conserved C-terminal domain oriented to the cytoplasm. Fabp5 is a cytoplasmic protein that binds long-chain fatty acids and other lipophilic ligands. Using a yeast two-hybrid screen, immunoprecipitation, microscale thermophoresis analysis and cellular fractionation, we discovered that Fabp5 interacts with the calnexin cytoplasmic C-tail domain at the endoplasmic reticulum. These observations identify Fabp5 as a previously unrecognized calnexin binding partner.

3.2840 **Heterogeneity in non-epitope loop sequence and outer membrane protein complexes alters antibody binding to the major porin protein PorB in serogroup B Neisseria meningitides**
Matthias, K.A., Strader, M.B., Nawar, H., Gao, Y.S., Lee, J., Patel, D.S., Im, W. and Bash, M.C.
Mol. Microbiol., ***105(6)***, *934-053 (2017)*

PorB is a well-characterized outer membrane protein that is common among *Neisseria* species and is required for survival. A vaccine candidate, PorB induces antibody responses that are directed against six variable surface-exposed loops that differ in sequence depending on serotype. Although *Neisseria meningitidis* is naturally competent and *porB* genetic mosaicism provides evidence for strong positive

selection, the sequences of PorB serotypes commonly associated with invasive disease are often conserved, calling into question the interaction of specific PorB loop sequences in immune engagement. In this report, we provide evidence that antibody binding to a PorB epitope can be altered by sequence mutations in non-epitope loops. Through the construction of hybrid PorB types and PorB molecular dynamics simulations, we demonstrate that loops both adjacent and non-adjacent to the epitope loop can enhance or diminish antibody binding, a phenotype that correlates with serum bactericidal activity. We further examine the interaction of PorB with outer membrane-associated proteins, including PorA and RmpM. Deletion of these proteins alters the composition of PorB-containing native complexes and reduces antibody binding and serum killing relative to the parental strain, suggesting that both intramolecular and intermolecular PorB interactions contribute to host adaptive immune evasion.

3.2841 Skewing of the population balance of lymphoid and myeloid cells by secreted and intracellular osteopontin

Kanayama, M., Xu, S., Danzaki, K., Gibson, J.R., Inoue, M., Gregory, S.G. and Shinohara, M.L.
Nature Immunol., ***18(9)***, *973-984 (2017)*

The balance of myeloid populations and lymphoid populations must be well controlled. Here we found that osteopontin (OPN) skewed this balance during pathogenic conditions such as infection and autoimmunity. Notably, two isoforms of OPN exerted distinct effects in shifting this balance through cell-type-specific regulation of apoptosis. Intracellular OPN (iOPN) diminished the population size of myeloid progenitor cells and myeloid cells, and secreted OPN (sOPN) increase the population size of lymphoid cells. The total effect of OPN on skewing the leukocyte population balance was observed as host sensitivity to early systemic infection with Candida albicans and T cell–mediated colitis. Our study suggests previously unknown detrimental roles for two OPN isoforms in causing the imbalance of leukocyte populations.

3.2842 TRIM37, a novel E3 ligase for PEX5-mediated peroxisomal matrix protein import

Wang, W., Xia, Z-J., Farre, J-C. and Subramami, S.
J. Cell Biol., ***216(9)***, *2843-2858 (2017)*

Most proteins destined for the peroxisomal matrix depend on the peroxisomal targeting signals (PTSs), which require the PTS receptor PEX5, whose deficiency causes fatal human peroxisomal biogenesis disorders (PBDs). *TRIM37* gene mutations cause muscle–liver–brain–eye (mulibrey) nanism. We found that TRIM37 localizes in peroxisomal membranes and ubiquitylates PEX5 at K464 by interacting with its C-terminal 51 amino acids (CT51), which is required for PTS protein import. PEX5 mutations (K464A or ΔCT51), or TRIM37 depletion or mutation, reduce PEX5 abundance by promoting its proteasomal degradation, thereby impairing its functions in cargo binding and PTS protein import in human cells. TRIM37 or PEX5 depletion induces apoptosis and enhances sensitivity to oxidative stress, underscoring the cellular requirement for functional peroxisomes. Therefore, TRIM37-mediated ubiquitylation stabilizes PEX5 and promotes peroxisomal matrix protein import, suggesting that mulibrey nanism is a new PBD.

3.2843 Placental Exosomes as Early Biomarker of Preeclampsia: Potential Role of Exosomal MicroRNAs Across Gestation

Salomon, C., Guanzon, D., Scholz-Romero, K., Longo, S., Correa, P., Illanes, S.E. and Rice, G.E.
J. Clin. Endocrinol. Metab., ***102(9)***, *3182-3194 (2017)*

Context
There is a need to develop strategies for early prediction of patients who will develop preeclampsia (PE) to establish preventive strategies to reduce the prevalence and severity of the disease and their associated complications.
Objective
The objective of this study was to investigate whether exosomes and their microRNA cargo present in maternal circulation can be used as early biomarker for PE.
Design, Setting, Patients, and Interventions
A retrospective stratified study design was used to quantify total exosomes and placenta-derived exosomes present in maternal plasma of normal (n = 32 per time point) and PE (n = 15 per time point) pregnancies. Exosomes present in maternal circulation were determined by nanoparticle tracking analysis. An Illumina TruSeq® Small RNA Library Prep Kit was used to construct a small RNA library from exosomal RNA obtained from plasma samples.
Results

In presymptomatic women, who subsequently developed PE, the concentration of total exosomes and placenta-derived exosomes in maternal plasma was significantly greater than those observed in controls, throughout pregnancy. The area under the receiver operating characteristic curves for total exosome and placenta-derived exosome concentrations were 0.745 ± 0.094 and 0.829 ± 0.077, respectively. In total, over 300 microRNAs were identified in exosomes across gestation, where hsa-miR-486-1-5p and hsa-miR-486-2-5p were identified as the candidate microRNAs.
Conclusions
Although the role of exosomes during PE remains to be fully elucidated, we suggest that the concentration and content of exosomes may be of diagnostic utility for women at risk for developing PE.

3.2844 Initial autophagic protection switches to disruption of autophagic flux by lysosomal instability during cadmium stress accrual in renal NRK-52E cells
Lee, W-K., Probst, S., Santoyo-Sanchez, M.P., Al-Hamdani, W., Diebels, I., von Sivers, J.K., Kerek, E., Prenner, E.J. and Thevenod, F.
Arch. Toxicol., ***91(10)***, *3225-3245 (2017)*

The renal proximal tubule (PT) is the major target of cadmium (Cd^{2+}) toxicity where Cd^{2+} causes stress and apoptosis. Autophagy is induced by cell stress, e.g., endoplasmic reticulum (ER) stress, and may contribute to cell survival or death. The role of autophagy in Cd^{2+}-induced nephrotoxicity remains unsettled due to contradictory results and lack of evidence for autophagic machinery damage by Cd^{2+}. Cd^{2+}-induced autophagy in rat kidney PT cell line NRK-52E and its role in cell death was investigated. Increased LC3-II and decreased p62 as autophagy markers indicate rapid induction of autophagic flux by Cd^{2+} (5–10 μM) after 1 h, accompanied by ER stress (increased p-PERK, p-eIF2α, CHOP). Cd^{2+} exposure exceeding 3 h results in p62/LC3-II accumulation, but diminished effect of lysosomal inhibitors (bafilomycin A1, pepstatin A +E-64d) on p62/LC3-II levels, indicating decreased autophagic flux and cargo degradation. At 24 h exposure, Cd^{2+} (5–25 μM) activates intrinsic apoptotic pathways (Bax/Bcl-2, PARP-1), which is not evident earlier (≤6 h) although cell viability by MTT assay is decreased. Autophagy inducer rapamycin (100 nM) does not overcome autophagy inhibition or Cd^{2+}-induced cell viability loss. The autophagosome–lysosome fusion inhibitor liensinine (5 μM) increases CHOP and Bax/Bcl-2-dependent apoptosis by low Cd^{2+} stress, but not by high Cd^{2+}. Lysosomal instability by Cd^{2+} (5 μM; 6 h) is indicated by increases in cellular sphingomyelin and membrane fluidity and decreases in cathepsins and LAMP1. The data suggest dual and temporal impact of Cd^{2+} on autophagy: Low Cd^{2+} stress rapidly activates autophagy counteracting damage but Cd^{2+} stress accrual disrupts autophagic flux and lysosomal stability, possibly resulting in lysosomal cell death.

3.2845 5-hydroxymethylcytosine accumulation in postmitotic neurons results in functional demethylation of expressed genes
Mellen, M., Ayata, P. and Heintz, N.
PNAS, ***114(37)***, *E7812-E7821 (2017)*

5-hydroxymethylcytosine (5hmC) occurs at maximal levels in postmitotic neurons, where its accumulation is cell-specific and correlated with gene expression. Here we demonstrate that the distribution of 5hmC in CG and non-CG dinucleotides is distinct and that it reflects the binding specificity and genome occupancy of methylcytosine binding protein 2 (MeCP2). In expressed gene bodies, accumulation of 5hmCG acts in opposition to 5mCG, resulting in "functional" demethylation and diminished MeCP2 binding, thus facilitating transcription. Non-CG hydroxymethylation occurs predominantly in CA dinucleotides (5hmCA) and it accumulates in regions flanking active enhancers. In these domains, oxidation of 5mCA to 5hmCA does not alter MeCP2 binding or expression of adjacent genes. We conclude that the role of 5-hydroxymethylcytosine in postmitotic neurons is to functionally demethylate expressed gene bodies while retaining the role of MeCP2 in chromatin organization.

3.2846 TrkB neurotrophic activities are blocked by α-synuclein, triggering dopaminergic cell death in Parkinson's disease
Kang, S.S., Zhang, Z., Liu, X., Manfredsson, F.P., Benskey, M.J., Cao, Z., Xu, J., Sun, Y.E. and Ye, K.
PNAS, ***114(40)***, *10773-10778 (2017)*

BDNF/TrkB neurotrophic signaling is essential for dopaminergic neuronal survival, and the activities are reduced in the substantial nigra (SN) of Parkinson's disease (PD). However, whether α-Syn (alpha-synuclein) aggregation, a hallmark in the remaining SN neurons in PD, accounts for the neurotrophic inhibition remains elusive. Here we show that α-Syn selectively interacts with TrkB receptors and inhibits

BDNF/TrkB signaling, leading to dopaminergic neuronal death. α-Syn binds to the kinase domain on TrkB, which is negatively regulated by BDNF or Fyn tyrosine kinase. Interestingly, α-Syn represses TrkB lipid raft distribution, decreases its internalization, and reduces its axonal trafficking. Moreover, α-Syn also reduces TrkB protein levels via up-regulation of TrkB ubiquitination. Remarkably, dopamine's metabolite 3,4-Dihydroxyphenylacetaldehyde (DOPAL) stimulates the interaction between α-Syn and TrkB. Accordingly, MAO-B inhibitor rasagiline disrupts α-Syn/TrkB complex and rescues TrkB neurotrophic signaling, preventing α-Syn–induced dopaminergic neuronal death and restoring motor functions. Hence, our findings demonstrate a noble pathological role of α-Syn in antagonizing neurotrophic signaling, providing a molecular mechanism that accounts for its neurotoxicity in PD.

3.2847 Organelle-specific single-molecule imaging of α4β2 nicotinic receptors reveals the effect of nicotine on receptor assembly and cell-surface trafficking
Fox-Loe, A.M., Moonschi, F.H. and Richrads, C.I.
J. Biol. Chem., ***292(51)***, *21159-21169 (2017)*

Nicotinic acetylcholine receptors (nAChRs) assemble in the endoplasmic reticulum (ER) and traffic to the cell surface as pentamers composed of α and β subunits. Many nAChR subtypes can assemble with varying subunit ratios, giving rise to multiple stoichiometries exhibiting different subcellular localization and functional properties. In addition to the endogenous neurotransmitter acetylcholine, nicotine also binds and activates nAChRs and influences their trafficking and expression on the cell surface. Currently, no available technique can specifically elucidate the stoichiometry of nAChRs in the ER *versus* those in the plasma membrane. Here, we report a method involving single-molecule fluorescence measurements to determine the structural properties of these membrane proteins after isolation in nanoscale vesicles derived from specific organelles. These cell-derived nanovesicles allowed us to separate single membrane receptors while maintaining them in their physiological environment. Sorting the vesicles according to the organelle of origin enabled us to determine localized differences in receptor structural properties, structural influence on transport between organelles, and changes in receptor assembly within intracellular organelles. These organelle-specific nanovesicles revealed that one structural isoform of the α4β2 nAChR was preferentially trafficked to the cell surface. Moreover, nicotine altered nAChR assembly in the ER, resulting in increased production of the receptor isoform that traffics more efficiently to the cell surface. We conclude that the combined effects of the increased assembly of one nAChR stoichiometry and its preferential trafficking likely drive the up-regulation of nAChRs on the cell surface upon nicotine exposure.

3.2848 Cell-Engineered Nanovesicle as a Surrogate Inducer of Contact-Dependent Stimuli
Kim, J., Han, C., Jo, W., Kang, S., Cho, S., Jeong, D., Gho, Y.S. and Park, J.
Adv. Healthcare Mater., ***6(17)***, *1700381 (2017)*

Heterotypic interactions between cells are crucial in various biological phenomena. Particularly, stimuli that regulate embryonic stem cell (ESC) fate are often provided from neighboring cells. However, except for feeder cultures, no practical methods are identified that can provide ESCs with contact-dependent cell stimuli. To induce contact-dependent cell stimuli in the absence of living cells, a novel method that utilizes cell-engineered nanovesicles (CNVs) that are made by extruding living cells through microporous membranes is described. Protein compositions of CNVs are similar to their originating cells, as well as freely diffusible and precisely scalable. Treatment of CNVs produced from three different stromal cells successfully induces the same effect as feeder cultures. The results suggest that the effects of CNVs are mainly mediated by membrane-associated components. The use of CNVs might constitute a novel and efficient tool for ESC research.

3.2849 Mammalian mitochondrial RNAs are degraded in the mitochondrial intermembrane space by RNASET2
Liu, P., Huang, J., Zheng, Q., Xie, l., Lu, X., Jin, J. and Wang, G.
Protein & Cell, ***8(10)***, *735-749 (2017)*

Mammalian mitochondrial genome encodes a small set of tRNAs, rRNAs, and mRNAs. The RNA synthesis process has been well characterized. How the RNAs are degraded, however, is poorly understood. It was long assumed that the degradation happens in the matrix where transcription and translation machineries reside. Here we show that contrary to the assumption, mammalian mitochondrial RNA degradation occurs in the mitochondrial intermembrane space (IMS) and the IMS-localized RNASET2 is the enzyme that degrades the RNAs. This provides a new paradigm for understanding mitochondrial RNA metabolism and transport.

3.2850 **Modulation of Autophagy by BDNF Underlies Synaptic Plasticity**
Nikoletopoulou, V., Sidiropoulou, K., Kallergi, E., Dalezios, Y. and Tavernarakis, N.
Cell Metabolism, ***26***, *230-242 (2017)*

Autophagy is crucial for neuronal integrity. Loss of key autophagic components leads to progressive neurodegeneration and structural defects in pre- and postsynaptic morphologies. However, the molecular mechanisms regulating autophagy in the brain remain elusive. Similarly, while it is widely accepted that protein turnover is required for synaptic plasticity, the contribution of autophagy to the degradation of synaptic proteins is unknown. Here, we report that BDNF signaling via the tropomyosin receptor kinase B (TrkB) and the phosphatidylinositol-3′ kinase (PI3K)/Akt pathway suppresses autophagy *in vivo*. In addition, we demonstrate that suppression of autophagy is required for BDNF-induced synaptic plasticity and for memory enhancement under conditions of nutritional stress. Finally, we identify three key remodelers of postsynaptic densities as cargo of autophagy. Our results establish autophagy as a pivotal component of BDNF signaling, which is essential for BDNF-induced synaptic plasticity. This molecular mechanism underlies behavioral adaptations that increase fitness in times of scarcity.

3.2851 **Expression and Subcellular Localization of the Kaposi's Sarcoma-Associated Herpesvirus K15P Protein during Latency and Lytic Reactivation in Primary Effusion Lymphoma Cells**
Smith, C.G., Kharkwal, H. and Wilson, D.W.
J. Virol., ***91(21)***, *e01370-17 (2017)*

The K15P membrane protein of Kaposi's sarcoma-associated herpesvirus (KSHV) interacts with multiple cellular signaling pathways and is thought to play key roles in KSHV-associated endothelial cell angiogenesis, regulation of B-cell receptor (BCR) signaling, and the survival, activation, and proliferation of BCR-negative primary effusion lymphoma (PEL) cells. Although full-length K15P is ~45 kDa, numerous lower-molecular-weight forms of the protein exist as a result of differential splicing and poorly characterized posttranslational processing. K15P has been reported to localize to numerous subcellular organelles in heterologous expression studies, but there are limited data concerning the sorting of K15P in KSHV-infected cells. The relationships between the various molecular weight forms of K15P, their subcellular distribution, and how these may differ in latent and lytic KSHV infections are poorly understood. Here we report that a cDNA encoding a full-length, ~45-kDa K15P reporter protein is expressed as an ~23- to 24-kDa species that colocalizes with the *trans*-Golgi network (TGN) marker TGN46 in KSHV-infected PEL cells. Following lytic reactivation by sodium butyrate, the levels of the ~23- to 24-kDa protein diminish, and the full-length, ~45-kDa K15P protein accumulates. This is accompanied by apparent fragmentation of the TGN and redistribution of K15P to a dispersed peripheral location. Similar results were seen when lytic reactivation was stimulated by the KSHV protein replication and transcription activator (RTA) and during spontaneous reactivation. We speculate that expression of different molecular weight forms of K15P in distinct cellular locations reflects the alternative demands placed upon the protein in the latent and lytic phases.

3.2852 **Expression and Characterization of Membrane-Type 4 Matrix Metalloproteinase (MT4-MMP) and its Different Forms in Melanoma**
Hieronimus, B., Pfohl, J., Busch, C. and Graeve, L.
Cell. Physiol. Biochem., ***42***, *198-210 (2017)*

Background/Aims: Membrane-type matrix metalloproteinases (MT-MMPs) are expressed on the cell surface and hydrolyze extracellular matrix components and signaling molecules by which they influence cancer cell migration and metastasis. Two of the six known MT-MMPs are anchored to the plasma membrane via a GPI anchor, one of which is MT4-MMP. Only little is known about MT4-MMP expression, synthesis, regulation and degradation. ***Methods:*** We analyzed several human cancer cell lines as well as tissue homogenates using Western blotting and quantitative PCR for the expression of MT4-MMP. Organelles of SK-Mel-28 cells were separated using continuous Iodixanol gradients. Glycosylation of the SK-Mel-28 protein was studied via glucosidases and site directed mutagenesis of the MT4-MMP cDNA prior to transfection. ***Results:*** We found the MT4-MMP highly expressed in human melanoma cell lines as well as skin and melanoma tissue samples. Three forms of MT4-MMP with molecular masses of 45 kDa, 58 kDa and 69 kDa were detected. Further, we demonstrate that the 58 kDa form is the mature protein in the cell membrane, while the 69 kDa form is its precursor found in intracellular compartments. The 69 kDa forms are processed by furin cleavage in the Golgi apparatus. Moreover, we identified Asn^{318}

as the single N-glycosylation site of MT4-MMP. ***Conclusion:*** We demonstrate the novel expression of MT4-MMP in melanocytic tissues and propose a precursor/product-relationship of the different forms of MT4-MMP in melanoma cells.

3.2853 Organelle membrane derived patches: reshaping classical methods for new targets
Shapalov, G., Ritaine, A., Bidaux, G., Slomianny, C., Borowiec, A-S., Gordienko, D., Bultynck, G., Skryma, R. and Prevarskaya, N.
*Scientific Reports, **7**:14082 (2017)*

Intracellular ion channels are involved in multiple signaling processes, including such crucial ones as regulation of cellular motility and fate. With 95% of the cellular membrane belonging to intracellular organelles, it is hard to overestimate the importance of intracellular ion channels. Multiple studies have been performed on these channels over the years, however, a unified approach allowing not only to characterize their activity but also to study their regulation by partner proteins, analogous to the patch clamp "golden standard", is lacking. Here, we present a universal approach that combines the extraction of intracellular membrane fractions with the preparation of patchable substrates that allows to characterize these channels in endogenous protein environment and to study their regulation by partner proteins. We validate this method by characterizing activity of multiple intracellular ion channels localized to different organelles and by providing detailed electrophysiological characterization of the regulation of IP_3R activity by endogenous Bcl-2. Thus, after synthesis and reshaping of the well-established approaches, organelle membrane derived patch clamp provides the means to assess ion channels from arbitrary cellular membranes at the single channel level.

3.2854 Exenatide Prevents Morphological and Structural Changes of Mitochondria Following Ischaemia-Reperfusion Injury
Lee, K.H., Ha, S.J., Woo, J-S., Lee, G-J., Lee, S-R., Kim, J.W., Park, H.K. and Kim, W.
*Heart, Lung and Circulation, **26**, 519-523 (2017)*

Background
Exenatide exerts cardioprotective effects by attenuating ischaemic reperfusion (IR) injury, possibly through activating the opening of mitochondrial ATP-sensitive potassium channels. We used atomic force microscopy (AFM) to investigate changes in mitochondrial morphology and properties in order to assess exenatide-mediated cardioprotection in IR injury.
Methods
We used an *in vivo* Sprague-Dawley rat IR model and *ex vivo* Langendorff injury model. In the left anterior descending artery (LAD) occlusion model, animals were randomly divided into three groups: sham-operated rats (Sham, n = 5), IR-injured rats treated with placebo (IR, n = 6), and IR-injured treated with exenatide (IR + EXE, n = 6). For the Langendorff model, rats were randomly divided into two groups: IR injury with placebo (IR, n = 4) and IR injury with exenatide (IR+EXE, n = 4). Morphological and mechanical changes of mitochondria were analysed by AFM.
Results
Exenatide pre-treatment improved cardiac function as evidenced by improvement in echocardiographic results. The ratio of infarct area (IA) to risk area (RA) was significantly reduced in exenatide-treated rats. According to AFM, IR significantly increased the area of isolated mitochondria, indicative of mitochondrial swelling. Treatment with exenatide reduced the mitochondrial area and ameliorated the adhesion force of mitochondrial surfaces.
Conclusions
Exenatide pre-treatment improves morphological and mechanical characteristics of mitochondria in response to IR injury in a rat model. These alterations in mitochondrial characteristics appear to play a cardioprotective role against IR injury.

3.2855 Extracellular vesicles as emerging targets in cancer: Recent development from bench to bedside
Wu, K., Xing, F., Wu, S-Y. and Watabe, K.
*BBA – Review on Cancer, **1868**. 538-563 (2017)*

Extracellular vesicles (EVs) have emerged as important players of cancer initiation and progression through cell-cell communication. They have been recognized as critical mediators of extracellular communications, which promote transformation, growth invasion, and drug-resistance of cancer cells. Interestingly, the secretion and uptake of EVs are regulated in a more controlled manner than previously anticipated. EVs are classified into three groups, (i) exosomes, (ii) microvesicles (MVs), and (iii) apoptotic

bodies (ABs), based on their sizes and origins, and novel technologies to isolate and distinguish these EVs are evolving. The biologically functional molecules harbored in these EVs, including nucleic acids, lipids, and proteins, have been shown to induce key signaling pathways in both tumor and tumor microenvironment (TME) cells for exacerbating tumor development. While tumor cell-derived EVs are capable of reprogramming stromal cells to generate a proper tumor cell niche, stromal-derived EVs profoundly affect the growth, resistance, and stem cell properties of tumor cells. This review summarizes and discusses these reciprocal communications through EVs in different types of cancers. Further understanding of the pathophysiological roles of different EVs in tumor progression is expected to lead to the discovery of novel biomarkers in liquid biopsy and development of tumor specific therapeutics. This review will also discuss the translational aspects of EVs and therapeutic opportunities of utilizing EVs in different cancer types.

3.2856 Adipose Tissue Macrophage-Derived Exosomal miRNAs Can Modulate *In Vivo* and *In Vitro* Insulin Sensitivity
Ying, W., Riopel, M., Bandyopadhyay, G., Dong, Y., Birmingham, A., Seo, J.B., Ofrecio, J.M., Wollam, J., Hernandez-Carretero, A., Fu, W., Li, P. and Olefsky, J.M.
*Cell, **171(2)**, 372-384 (2017)*

MiRNAs are regulatory molecules that can be packaged into exosomes and secreted from cells. Here, we show that adipose tissue macrophages (ATMs) in obese mice secrete miRNA-containing exosomes (Exos), which cause glucose intolerance and insulin resistance when administered to lean mice. Conversely, ATM Exos obtained from lean mice improve glucose tolerance and insulin sensitivity when administered to obese recipients. miR-155 is one of the miRNAs overexpressed in obese ATM Exos, and earlier studies have shown that PPARγ is a miR-155 target. Our results show that miR-155KO animals are insulin sensitive and glucose tolerant compared to controls. Furthermore, transplantation of WT bone marrow into miR-155KO mice mitigated this phenotype. Taken together, these studies show that ATMs secrete exosomes containing miRNA cargo. These miRNAs can be transferred to insulin target cell types through mechanisms of paracrine or endocrine regulation with robust effects on cellular insulin action, *in vivo* insulin sensitivity, and overall glucose homeostasis.

3.2857 Sortilin limits EGFR signaling by promoting its internalization in lung cancer
Al-Akhrass, H., Naves, T., Vincent, F., Magnaudeix, A., Durand, K., Bertin, F., Melloni, B., Jauberteau, M-O. and Lalloue, F.
*Nature Communications, **8**:1182 (2017)*

Tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR) transduce information from the microenvironment into the cell and activate homeostatic signaling pathways. Internalization and degradation of EGFR after ligand binding limits the intensity of proliferative signaling, thereby helping to maintain cell integrity. In cancer cells, deregulation of EGFR trafficking has a variety of effects on tumor progression. Here we report that sortilin is a key regulator of EGFR internalization. Loss of sortilin in tumor cells promoted cell proliferation by sustaining EGFR signaling at the cell surface, ultimately accelerating tumor growth. In lung cancer patients, sortilin expression decreased with increased pathologic grade, and expression of sortilin was strongly correlated with survival, especially in patients with high EGFR expression. Sortilin is therefore a regulator of EGFR intracellular trafficking that promotes receptor internalization and limits signaling, which in turn impacts tumor growth.

3.2858 The lipid raft-dwelling protein US9 can be manipulated to target APP compartmentalization, APP processing, and neurodegenerative disease pathogenesis
Brandimarti, R., Hill, G.S., Geiger, J.D. and Meucci, O.
*Scientific Reports, **7**:15103 (2017)*

The trafficking behavior of the lipid raft-dwelling US9 protein from Herpes Simplex Virus strikingly overlaps with that of the amyloid precursor protein (APP). Both US9 and APP processing machinery rely on their ability to shuttle between endosomes and plasma membranes, as well as on their lateral accumulation in lipid rafts. Therefore, repurposing US9 to track/modify these molecular events represents a valid approach to investigate pathological states including Alzheimer's disease and HIV-associated neurocognitive disorders where APP misprocessing to amyloid beta formation has been observed. Accordingly, we investigated the cellular localization of US9-driven cargo in neurons and created a US9-driven functional assay based on the exogenous enzymatic activity of Tobacco Etch Virus Protease. Our results demonstrate that US9 can direct and control cleavage of recombinant proteins exposed on the

luminal leaflet of transport vesicles. Furthermore, we confirmed that US9 is associated with lipid-rafts and can target functional enzymes to membrane microdomains where pathologic APP-processing is thought to occur. Overall, our results suggest strongly that US9 can serve as a molecular driver that targets functional cargos to the APP machinery and can be used as a tool to study the contribution of lipid rafts to neurodegenerative disease conditions where amyloidogenesis has been implicated.

3.2859 Exosomes in Prion Diseases
Hartmann, A., Altmeppen, H., Krasemann, S. and Glatzel, M.
*Neuromethods, **129**, 197-207 (2017)*

Dementias are characterized by generation and tissue deposition of proteins altered in their secondary or tertiary structure. Prion diseases are prominent and well-studied examples of these diseases. Initiation of prion disease is associated to the conversion of the cellular prion protein (PrP^{C}) to its pathogenic isoform (PrP^{Sc}). Spread of PrP^{Sc} throughout the central nervous system leads to disease progression and is achieved by cell-to-cell transfer, axonal or nanotube-mediated transport or exosomes.
In this chapter we describe how to isolate, purify, and quality control exosomes, and provide helpful notes for practical guidance and troubleshooting in these techniques.

3.2860 Technical Aspects for the Evaluation of Exosomes and Their Content
Fontana, S., Giallombardo, M. and Alessandro, R.
Liquid Biopsy in Cancer Patients, 61-70 (2017)

Liquid biopsy is a precious source of exosomes, nanometer-sized vesicles (40–100 nm diameter) that play a relevant role in the cell-cell communication, strongly depending on the nature of the transported molecules (proteins, mRNAs, miRNAs, and lipids).
Since a significant body of literature has demonstrated that exosomes released by cancer cells carry tumor-specific RNAs and proteins, they are widely considered very attractive targets for diagnostic application. This chapter focuses on the isolation and study of exosomes from liquid biopsies and summarizes the recent exosomal miRNA and protein profiling data supporting the potential role of tumor-derived exosomes as biomarkers and their potential application in clinical settings.

3.2861 PIP1 aquaporins, sterols, and osmotic water permeability of plasma membranes from etiolated pea seedlings
Belugin, B.V., Zhestkova, I.M., Piotrovskii, M.S., Lapshin, N.K. and Trofimova, M.S.
*Biochemistry (Moscow), Suppl: Membrane and Cell Biol., **11(2)**, 168-176 (2017)*

Plasma membrane isolated from microsomal membranes of pea seedling root and shoot cells by means of aqueous two-phase polymer system was separated by flotation in discontinuous OptiPrep gradient into "light" ($\leq$1.146 g/cm^3) and "heavy" ($\geq$1.146 g/cm^3) fractions. Osmotic water permeability of plasma membrane and its two fractions was investigated by inducing transmembrane osmotic gradient on the vesicle membrane and recording the kinetics of vesicle osmotic shrinkage by the stopped-flow method. Rate constants of osmotic shrinkage and coefficients of osmotic water permeability of the membranes were estimated on the basis of the kinetic curve approximation by exponential dependencies and using electron microscope data on vesicles sizes. In plasma membrane and its fractions the content of sterols and PIP1 aquaporins was determined. It was found that in "light" PM fractions from both roots and shoots the content of PIP1 aquaporins and sterols was higher and the osmotic water permeability coefficient was lower than in "heavy" fractions of plasma membrane. The results indicate that plasma membrane of roots and shoots is heterogeneous in osmotic water permeability. This heterogeneity may be related with the presence of microdomains with different content of aquaporins and sterols in the membrane.

3.2862 Exploiting the Gastric Epithelial Barrier: Helicobacter pylori's Attack on Tight and Adherens Junctions
Backert, S., Schmidt, T.P., harrer, A. and Wessler, S.
*Current Topics in Microbiol. Immunol., **400**, 195-226 (2017)*

Highly organized intercellular tight and adherens junctions are crucial structural components for establishing and maintenance of epithelial barrier functions, which control the microbiota and protect against intruding pathogens in humans. Alterations in these complexes represent key events in the development and progression of multiple infectious diseases as well as various cancers. The gastric pathogen *Helicobacter pylori* exerts an amazing set of strategies to manipulate these epithelial cell-to-cell

junctions, which are implicated in changing cell polarity, migration and invasive growth as well as pro-inflammatory and proliferative responses. This chapter focuses on the *H. pylori* pathogenicity factors VacA, CagA, HtrA and urease, and how they can induce host cell signaling involved in altering cell-to-cell permeability. We propose a stepwise model for how *H. pylori* targets components of tight and adherens junctions in order to disrupt the gastric epithelial cell layer, giving fresh insights into the pathogenesis of this important bacterium.

3.2863 Bacteriophage Transcytosis Provides a Mechanism To Cross Epithelial Cell Layers
Nguyen, S., Baker, K., PAdman, M.S., Patwa, R., Dunstan, R.A., Weston, T.A., Schlosser, K., Bailey, B., Lithgow, T., Lazarou, M., Luzue, A., Rohwer,m F., Blumberg, R.S. and Barr, J.J.
*mBio, **8(6)**, e01874-17 (2017)*

viruses are among the most numerous biological entities within the human body. These viruses are found within regions of the body that have conventionally been considered sterile, including the blood, lymph, and organs. However, the primary mechanism that bacterial viruses use to bypass epithelial cell layers and access the body remains unknown. Here, we used *in vitro* studies to demonstrate the rapid and directional transcytosis of diverse bacteriophages across confluent cell layers originating from the gut, lung, liver, kidney, and brain. Bacteriophage transcytosis across cell layers had a significant preferential directionality for apical-to-basolateral transport, with approximately 0.1% of total bacteriophages applied being transcytosed over a 2-h period. Bacteriophages were capable of crossing the epithelial cell layer within 10 min with transport not significantly affected by the presence of bacterial endotoxins. Microscopy and cellular assays revealed that bacteriophages accessed both the vesicular and cytosolic compartments of the eukaryotic cell, with phage transcytosis suggested to traffic through the Golgi apparatus via the endomembrane system. Extrapolating from these results, we estimated that 31 billion bacteriophage particles are transcytosed across the epithelial cell layers of the gut into the average human body each day. The transcytosis of bacteriophages is a natural and ubiquitous process that provides a mechanistic explanation for the occurrence of phages within the body.

3.2864 Isolation of exosomes from whole blood by integrating acoustics and microfluidics
Wu, M., Ouyang, Y., Wang, Z., Zhang, R., Huang, P-H., Chen, C., Li, H., Li, P., Quinn, D., Dao, M., Suresh, S., Sadovsky, Y. and Huang, T.J.
*PNAS, **114(40)**, 10584-10589 (2017)*

Exosomes are nanoscale extracellular vesicles that play an important role in many biological processes, including intercellular communications, antigen presentation, and the transport of proteins, RNA, and other molecules. Recently there has been significant interest in exosome-related fundamental research, seeking new exosome-based biomarkers for health monitoring and disease diagnoses. Here, we report a separation method based on acoustofluidics (i.e., the integration of acoustics and microfluidics) to isolate exosomes directly from whole blood in a label-free and contact-free manner. This acoustofluidic platform consists of two modules: a microscale cell-removal module that first removes larger blood components, followed by extracellular vesicle subgroup separation in the exosome-isolation module. In the cell-removal module, we demonstrate the isolation of 110-nm particles from a mixture of micro- and nanosized particles with a yield greater than 99%. In the exosome-isolation module, we isolate exosomes from an extracellular vesicle mixture with a purity of 98.4%. Integrating the two acoustofluidic modules onto a single chip, we isolated exosomes from whole blood with a blood cell removal rate of over 99.999%. With its ability to perform rapid, biocompatible, label-free, contact-free, and continuous-flow exosome isolation, the integrated acoustofluidic device offers a unique approach to investigate the role of exosomes in the onset and progression of human diseases with potential applications in health monitoring, medical diagnosis, targeted drug delivery, and personalized medicine.

3.2865 A novel intracellular pool of LFA-1 is critical for asymmetric CD8+ T cell activation and differentiation
Capece, T., Walling, B.L., Lim, K., Kim, K-D., bae, S., Chung, H-L., Topham, D.J. and Kim, M.
*J. Cell Biol., **216(11)**, 3817-3829 (2017)*

The integrin lymphocyte function–associated antigen 1 (LFA-1; CD11a/CD18) is a key T cell adhesion receptor that mediates stable interactions with antigen-presenting cell (APC), as well as chemokine-mediated migration. Using our newly generated CD11a-mYFP knock-in mice, we discovered that naive $CD8^+$ T cells reserve a significant intracellular pool of LFA-1 in the uropod during migration. Intracellular LFA-1 quickly translocated to the cell surface with antigenic stimulus. Importantly, the redistribution of

intracellular LFA-1 at the contact with APC was maintained during cell division and led to an unequal inheritance of LFA-1 in divided T cells. The daughter $CD8^+$ T cells with disparate LFA-1 expression showed different patterns of migration on ICAM-1, APC interactions, and tissue retention, as well as altered effector functions. In addition, we identified Rab27 as an important regulator of the intracellular LFA-1 translocation. Collectively, our data demonstrate that an intracellular pool of LFA-1 in naive $CD8^+$ T cells plays a key role in T cell activation and differentiation.

3.2866 Nef is secreted in exosomes from Nef.GFP-expressing and HIV-1-infected human astrocytes
Diminkus, P.P., Ferdin, J., Plemenitas, A., Peterlin, B.M. and Lenassi, M.
J. Neurovirol., ***23***, *713-724 (2017)*

HIV-1 infection of the central nervous system causes HIV-associated neurocognitive disorders, even in aviremic patients. Although astrocyte malfunction was associated to these disorders, their implication is overshadowed by contributions of microglia and macrophages. Astrocytes are infected with HIV-1 in vivo and express a relevant amount of viral protein Nef. Nef was shown to stimulate its own release in exosomes from diverse cell types, which in turn have damaging effects on neighboring cells. Using immunoblotting and electron microscopy, we showed that human astrocytes expressing Nef.GFP similarly release Nef in exosomes. Importantly, Nef.GFP expression increases the secretion of exosomes from human astrocytes up to 5.5-fold, as determined by total protein content and nanoparticle tracking analysis. Protein analysis of exosomes and viruses separated on iodixanol gradient further showed that native or pseudotyped HIV-1-infected human astrocytes release exosomes, which contain Nef. Our results provide the basis for future studies of the damaging role of Nef-exosomes produced by HIV-infected astrocytes on the central nervous system.

3.2867 SG2NA is a regulator of endoplasmic reticulum (ER) homeostasis as its depletion leads to ER stress
Jain, B.P., Pandey, S., Saleem, N., tanti, G.T., Mishra, s. and Goswami, S.K:
Cell Stress and Chaperones, ***22***, *853-866 (2017)*

SG2NA belongs to a three-member striatin subfamily of WD40 repeat superfamily of proteins. It has multiple protein-protein interaction domains involved in assembling supramolecular signaling complexes. Earlier, we had demonstrated that there are at least five variants of SG2NA generated by alternative splicing, intron retention, and RNA editing. Such versatile and dynamic mode of regulation implicates it in tissue development. In order to shed light on its role in cell physiology, total proteome analysis was performed in NIH3T3 cells depleted of 78 kDa SG2NA, the only isoform expressing therein. A number of ER stress markers were among those modulated after knockdown of SG2NA. In cells treated with the ER stressors thapsigargin and tunicamycin, expression of SG2NA was increased at both mRNA and protein levels. The increased level of SG2NA was primarily in the mitochondria and the microsomes. A mouse injected with thapsigargin also had an increase in SG2NA in the liver but not in the brain. Cell cycle analysis suggested that while loss of SG2NA reduces the level of cyclin D1 and retains a population of cells in the G1 phase, concurrent ER stress facilitates their exit from G1 and traverse through subsequent phases with concomitant cell death. Thus, SG2NA is a component of intrinsic regulatory pathways that maintains ER homeostasis.

3.2868 Characterization of a novel role for the dynamin mechanoenzymes in the regulation of human sperm acrosomal exocytosis
Zhou, W., Anderson, A.L., Turner, A.P., De lullis, G.N., McCluskey, A., Mclaughlin, E.A. and Nixon, B.
Mol. Hum. Reprod., ***23(10)***, *657-673 (2017)*

STUDY QUESTION
Does dynamin regulate human sperm acrosomal exocytosis?
SUMMARY ANSWER
Our studies of dynamin localization and function have implicated this family of mechanoenzymes in the regulation of progesterone-induced acrosomal exocytosis in human spermatozoa.
WHAT IS KNOWN ALREADY
Completion of an acrosome reaction is a prerequisite for successful fertilization in all studied mammalian species. It follows that failure to complete this unique exocytotic event represents a common aetiology in the defective spermatozoa of male infertility patients that have failed IVF in a clinical setting. Recent studies have implicated the dynamin family of mechanoenzymes as important regulators of the acrosome reaction in murine spermatozoa. The biological basis of this activity appears to rest with the ability of dynamin to polymerize around newly formed membrane vesicles and subsequently regulate the rate of

fusion pore expansion. To date, however, the dynamin family of GTPases have not been studied in the spermatozoa of non-rodent species. Here, we have sought to examine the presence and functional significance of dynamin in human spermatozoa.
STUDY DESIGN, SIZE, DURATION
Dynamin expression was characterized in the testis and spermatozoa of several healthy normozoospermic individuals. In addition, we assessed the influence of selective dynamin inhibition on the competence of human spermatozoa to undergo a progesterone-induced acrosome reaction. A minimum of five biological and technical replicates were performed to investigate both inter- and intra-donor variability in dynamin expression and establish statistical significance in terms of the impact of dynamin inhibition.
PARTICIPANTS/MATERIALS, SETTING, METHODS
The expression and the localization of dynamin in the human testis, epididymis and mature spermatozoa were determined through the application of immunofluorescence, immunoblotting and/or electron microscopy. Human semen samples were fractionated via density gradient centrifugation and the resultant populations of good and poor quality spermatozoa were induced to capacitate and acrosome react in the presence or absence of selective dynamin inhibitors. The acrosome integrity of live spermatozoa was subsequently assessed via the use of fluorescently conjugated *Arachis hypogea* lectin (PNA). The influence of dynamin phosphorylation and the regulatory kinase(s) responsible for this modification in human spermatozoa were also assessed via the use of *in situ* proximity ligation assays and pharmacological inhibition. In all experiments, ≥100 spermatozoa were assessed/treatment group and all graphical data are presented as the mean values ± SEM, with statistical significance being determined by ANOVA.
MAIN RESULTS AND THE ROLE OF CHANCE
Dynamin 1 (DNM1) and DNM2, but not DNM3, were specifically localized to the acrosomal region of the head of human spermatozoa, an ideal position from which to regulate acrosomal exocytosis. In keeping with this notion, pharmacological inhibition of DNM1 and DNM2 was able to significantly suppress the rates of acrosomal exocytosis stimulated by progesterone. Furthermore, our comparison of dynamin expression in good and poor quality spermatozoa recovered from the same ejaculate, revealed a significant reduction in the amount of DNM2 in the latter subpopulation of cells. In contrast, DNM1 was detected at equivalent levels in both subpopulations of spermatozoa. Such findings are of potential significance given that the poor quality spermatozoa proved refractory to the induction of a progesterone stimulated acrosome reaction. In seeking to identify the regulatory influence of progesterone on DNM2 function, we were able to establish that the protein is a substrate for CDK1-dependent phosphorylation. The functional significance of DNM2 phosphorylation was illustrated by the fact that pharmacological inhibition of CDK1 elicited a concomitant suppression of both DNM2-Ser764 phosphorylation and the overall rates of progesterone-induced acrosomal exocytosis.

3.2869 Albuminuria is not associated with elevated urinary vesicle concentration but can confound nanoparticle tracking analysis

McNicholas, K., Li, J.Y., Michael, M.Z. and Gleadle, J.M:
*Nephrology, **22(11)**, 854-863 (2017)*

Aim
Extracellular vesicles, such as exosomes, are present in urine with reports of roles in intercellular signalling and diagnostic utility. However, the extent to which the concentration and characteristics of urinary vesicles are altered in albuminuric renal disease has not been well characterized. In this study, we examined the number and characteristics of extracellular vesicles in albuminuric urine.
Methods
Vesicles were isolated from the urine of 32 patients with varying levels of albuminuria using ultracentrifugation and density gradient purification and were examined using nanoparticle tracking analysis, immunoblotting and transmission electron microscopy. The size profile of particles in these urine preparations was compared with albumin-containing solutions.
Results
Overall, there were no substantial differences in the number, or characteristics, of vesicles released into proteinuric urine. Analysis of albumin-containing solutions showed particles of exosome-like size, suggesting that such particles can mimic exosomes in standard nanoparticle tracking analysis. Albumin and IgG depletion of proteinuric urine resulted in a substantial reduction in the concentration of particles detected by nanoparticle tracking analysis.
Conclusion
There was no increase in urinary vesicle concentration in patients with albuminuria. Furthermore, these results demonstrate the need for cautious interpretation of nanoparticle tracking analysis of vesicle

concentration in biological fluids containing protein and for sophisticated preparative methods in vesicle purification from urine.

3.2870 Proteomics Insights into Autophagy
Cudjoe, Jr., E.K., Saleh, T., Hawkridge, A.M. and Gewirtz, D.A.
*Proteomics, **17**, 1700022 (2017)*

Autophagy, a conserved cellular process by which cells recycle their contents either to maintain basal homeostasis or in response to external stimuli, has for the past two decades become one of the most studied physiological processes in cell biology. The 2016 Nobel Prize in Medicine and Biology awarded to Dr. Ohsumi Yoshinori, one of the first scientists to characterize this cellular mechanism, attests to its importance. The induction and consequent completion of the process of autophagy results in wide ranging changes to the cellular proteome as well as the secretome. MS-based proteomics affords the ability to measure, in an unbiased manner, the ubiquitous changes that occur when autophagy is initiated and progresses in the cell. The continuous improvements and advances in mass spectrometers, especially relating to ionization sources and detectors, coupled with advances in proteomics experimental design, has made it possible to study autophagy, among other process, in great detail. Innovative labeling strategies and protein separation techniques as well as complementary methods including immuno-capture/blotting/staining have been used in proteomics studies to provide more specific protein identification. In this review, we will discuss recent advances in proteomics studies focused on autophagy.

3.2871 Extracellular vesicles in the tumor microenvironment: Therapeutic resistance, clinical biomarkers, and targeting strategies
Han, L., Xu, J., Xu, Q., Zhang, B., lam, E.W-F. and Sun, Y.
*Med. Res. Rev., **37(6)**, 1318-1349 (2017)*

Numerous studies have proved that cell-nonautonomous regulation of neoplastic cells is a distinctive and essential characteristic of tumorigenesis. Two way communications between the tumor and the stroma, or within the tumor significantly influence disease progression and modify treatment responses. In the tumor microenvironment (TME), malignant cells utilize paracrine signaling initiated by adjacent stromal cells to acquire resistance against multiple types of anticancer therapies, wherein extracellular vesicles (EVs) substantially promote such events. EVs are nanoscaled particles enclosed by phospholipid bilayers, and can mediate intercellular communications between cancerous cells and the adjacent microenvironment to accelerate pathological proceeding. Here we review the most recent studies of EV biology and focus on key cell lineages of the TME and their EV cargoes that are biologically active and responsible for cancer resistance, including proteins, RNAs, and other potentially essential components. Since EVs are emerging as novel but critical elements in establishing and maintaining hallmarks of human cancer, timely and insightful understanding of their molecular properties and functional mechanisms would pave the road for clinical diagnosis, prognosis, and effective targeting in the global landscape of precision medicine. Further, we address the potential of EVs as promising biomarkers in cancer clinics and summarize the technical improvements in EV preparation, analysis, and imaging. We highlight the practical issues that should be exercised with caution to guide the development of targeting agents and therapeutic methodologies to minimize cancer resistance driven by EVs, thereby allowing to effectively control the early steps of disease exacerbation.

3.2872 Accumulation of undegraded autophagosomes by expression of dominant-negative STX17 (syntaxin 17) mutants
Uematsu, M., Nishimura, T., Sakamaki, Y., Yamamoto, H. and Mizushima, N.
*Autophagy, **13(8)**, 1452-1464 (2017)*

Macroautophagy/autophagy, which is one of the main degradation systems in the cell, is mediated by a specialized organelle, the autophagosome. Purification of autophagosomes before fusion with lysosomes is important for both mechanistic and physiological studies of the autophagosome. Here, we report a simple method to accumulate undigested autophagosomes. Overexpression of the autophagosomal Qa-SNARE STX17 (syntaxin 17) lacking the N-terminal domain (NTD) or N-terminally tagged GFP-STX17 causes accumulation of autophagosomes. A HeLa cell line, which expresses GFP-STX17ΔNTD or full-length GFP-STX17 under the control of the tetracycline-responsive promoter, accumulates a large number of undigested autophagosomes devoid of lysosomal markers or early autophagy factors upon treatment with doxycycline. Using this inducible cell line, nascent autophagosomes can be easily purified by OptiPrep

density-gradient centrifugation and immunoprecipitation. This novel method should be useful for further characterization of nascent autophagosomes.

3.2873 Targeting of Cellular Organelles by Fluorescent Plasmid DNA Nanoparticles
Costa, D., Costa, C., Caldeira, M., Cortes, L., Queiroz, J.A. and Cruz, C.
*Biomacromolecules, **18(9)**, 2928-2936 (2017)*

The development of a suitable delivery system and the targeting of intracellular organelles are both essential for the success of drug and gene therapies. The conception of fluorescent ligands, displaying targeting specificity together with low toxicity, is an emerging and reliable tool to develop innovative delivery systems. Biocompatible BSA or pDNA/ligand nanoparticles were synthesized by a coprecipitation method and were shown to display adequate sizes and morphology for delivery purposes, and positive surface charges. Additionally, these fluorescent vectors can target specific intracellular organelles. In vitro transfection mediated by BSA or pDNA based carriers can result in the accumulation of BSA in the cytosol, lysosomes, and mitochondria or the expression of the plasmid-encoded protein, respectively. Moreover, the therapeutic effect of pDNA/ligand vectors in cancer gene therapy instigates further research aiming clinical translation.

3.2874 Renal Regenerative Potential of Different Extracellular Vesicle Populations Derived from Bone Marrow Mesenchymal Stromal Cells free access
Bruno, S., Tapparo, M., Collino, F., Chiabotto, G., Deregibus, M.C., Lindoso, R.S., Neri, F., Kholia, S., Giunti, S., Wen, S., Qusenberry, P. and Camussi, G.
*Tissue Engineering: Part A, **23(21-22)**, 1262-1273 (2017)*

Extracellular vesicles (EVs) derived from human bone marrow mesenchymal stromal cells (MSCs) promote the regeneration of kidneys in different animal models of acute kidney injury (AKI) in a manner comparable with the cells of origin. However, due to the heterogeneity observed in the EVs isolated from MSCs, it is unclear which population is responsible for the proregenerative effects. We therefore evaluated the effect of various EV populations separated by differential ultracentrifugation (10K population enriched with microvesicles and 100K population enriched with exosomes) on AKI recovery. Only the exosomal-enriched population induced an improvement of renal function and morphology comparable with that of the total EV population. Interestingly, the 100K EVs exerted a proproliferative effect on murine tubular epithelial cells, both *in vitro* and *in vivo*. Analysis of the molecular content from the different EV populations revealed a distinct profile. The 100K population, for instance, was enriched in specific mRNAs (CCNB1, CDK8, CDC6) reported to influence cell cycle entry and progression; miRNAs involved in regulating proliferative/antiapoptotic pathways and growth factors (hepatocyte growth factor and insulin-like growth factor-1) that could explain the effect of renal tubular cell proliferation. On the other hand, the EV population enriched in microvesicles (10K) was unable to induce renal regeneration and had a molecular profile with lower expression of proproliferative molecules. In conclusion, the different molecular composition of exosome- and microvesicle-enriched populations may explain the regenerative effect of EVs observed in AKI.

3.2875 Phosphorylation of LAMP2A by p38 MAPK couples ER stress to chaperone-mediated autophagy
Li, W., Zhu, J., Dou, J., She, H., tao, K., Xu, H., yang, Q. and Mao, Z.
*Nature Communications, **8**:1763 (2017)*

Endoplasmic reticulum (ER) and lysosomes coordinate a network of key cellular processes including unfolded protein response (UPR) and autophagy in response to stress. How ER stress is signaled to lysosomes remains elusive. Here we find that ER disturbance activates chaperone-mediated autophagy (CMA). ER stressors lead to a PERK-dependent activation and recruitment of MKK4 to lysosomes, activating p38 MAPK at lysosomes. Lysosomal p38 MAPK directly phosphorylates the CMA receptor LAMP2A at T211 and T213, which causes its membrane accumulation and active conformational change, activating CMA. Loss of ER stress-induced CMA activation sensitizes cells to ER stress-induced death. Neurotoxins associated with Parkinson's disease fully engages ER-p38 MAPK–CMA pathway in the mouse brain and uncoupling it results in a greater loss of SNc dopaminergic neurons. This work identifies the coupling of ER and CMA as a critical regulatory axis fundamental for physiological and pathological stress response.

3.2876 A subset of extracellular vesicles carries the bulk of microRNAs in commercial dairy cow's milk
Benmoussa, A., Ly, S., Shan, S.T., Laugier, J., Boilard, E., Gilbert, C. and Provost, P.

*J. Extracellular Vesicles, **6**, 1401897 (2017)*

MicroRNAs are small gene-regulatory RNAs that are found in various biological fluids, including milk, where they are often contained inside extracellular vesicles (EVs), like exosomes. In a previous study, we reported that commercial dairy cow's milk microRNAs resisted simulated digestion and were not exclusively associated with canonical exosomes. Here, we report the characterization of a milk EV subset that sediments at lower ultracentrifugation speeds and that contains the bulk of microRNAs. Milk EVs were isolated by differential ultracentrifugation and Iodixanol density gradient (IDG), and analysed for (1) microRNA enrichment by reverse transcription and quantitative polymerase chain reaction (RT-qPCR), and (2) EV-associated proteins by Western blot. Milk EVs were characterized further by dynamic light scattering (DLS), density measurements, fluorescent DiR and RNA labelling, high-sensitivity flow cytometry (HS-FCM), transmission electron microscopy (TEM), proteinase K and RNase A assay, and liquid chromatography tandem-mass spectrometry (LC-MS/MS). We found that the bulk of milk microRNAs (e.g., bta-miR-125b, bta-miR-148a, etc.) sediment at 12,000 *g* and 35,000 *g*. Their distribution pattern was different from that of exosome-enriched proteins, but similar to that of several proteins commonly found in milk fat globule membranes (MFGM), including xanthine dehydrogenase (XDH). These low-speed ultracentrifugation pellets contained cytoplasm-enclosing phospholipid bilayered membrane vesicles of a density comprised between 1.11 and 1.14 g/mL in Iodixanol. This milk EV subset of ~100 nm in diameter/~200 nm hydrodynamic size resisted to proteinase K digestion and protected their microRNA content from RNase A digestion. Our results support the existence of a milk EV subset pelleting at low ultracentrifugations speeds, with a protein coating comparable with MFGM, which contains and protects the bulk of milk microRNAs from degradation. This milk EV subset may represent a new EV population of interest, whose content in microRNAs and proteins supports its potential bioactivity.

3.2877 ORAI channels are critical for receptor-mediated endocytosis of albumin
Zeng, B. et al
*Nature Communications, **8**:1920 (2017)*

Impaired albumin reabsorption by proximal tubular epithelial cells (PTECs) has been highlighted in diabetic nephropathy (DN), but little is known about the underlying molecular mechanisms. Here we find that ORAI1-3, are preferentially expressed in PTECs and downregulated in patients with DN. Hyperglycemia or blockade of insulin signaling reduces the expression of ORAI1-3. Inhibition of ORAI channels by BTP2 and diethylstilbestrol or silencing of ORAI expression impairs albumin uptake. Transgenic mice expressing a dominant-negative Orai1 mutant (E108Q) increases albuminuria, and in vivo injection of BTP2 exacerbates albuminuria in streptozotocin-induced and Akita diabetic mice. The albumin endocytosis is Ca2+-dependent and accompanied by ORAI1 internalization. Amnionless (AMN) associates with ORAIs and forms STIM/ORAI/AMN complexes after Ca2+ store depletion. STIM1/ORAI1 colocalizes with clathrin, but not with caveolin, at the apical membrane of PTECs, which determines clathrin-mediated endocytosis. These findings provide insights into the mechanisms of protein reabsorption and potential targets for treating diabetic proteinuria.

3.2878 Malaria parasite DNA-harbouring vesicles activate cytosolic immune sensors
Sisquella, X. et al
*Nature Communications, **8**, 1985 (2017)*

STING is an innate immune cytosolic adaptor for DNA sensors that engage malaria parasite (*Plasmodium falciparum*) or other pathogen DNA. As *P. falciparum* infects red blood cells and not leukocytes, how parasite DNA reaches such host cytosolic DNA sensors in immune cells is unclear. Here we show that malaria parasites inside red blood cells can engage host cytosolic innate immune cell receptors from a distance by secreting extracellular vesicles (EV) containing parasitic small RNA and genomic DNA. Upon internalization of DNA-harboring EVs by human monocytes, *P. falciparum* DNA is released within the host cell cytosol, leading to STING-dependent DNA sensing. STING subsequently activates the kinase TBK1, which phosphorylates the transcription factor IRF3, causing IRF3 to translocate to the nucleus and induce STING-dependent gene expression. This DNA-sensing pathway may be an important decoy mechanism to promote *P. falciparum* virulence and thereby may affect future strategies to treat malaria.

3.2879 Endocytosis regulates TDP-43 toxicity and turnover
Liu, G., Coyne, A.N., Pei, F., Vaughan, S., Chaung, M., Zarnescu, D.C. and Buchan, J.R.
*Nature Communications, **8**:2092 (2017)*

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron degenerative disease. ALS-affected motor neurons exhibit aberrant localization of a nuclear RNA binding protein, TDP-43, into cytoplasmic aggregates, which contributes to pathology via unclear mechanisms. Here, we demonstrate that TDP-43 turnover and toxicity depend in part upon the endocytosis pathway. TDP-43 inhibits endocytosis, and co-localizes strongly with endocytic proteins, including in ALS patient tissue. Impairing endocytosis increases TDP-43 toxicity, aggregation, and protein levels, whereas enhancing endocytosis reverses these phenotypes. Locomotor dysfunction in a TDP-43 ALS fly model is also exacerbated and suppressed by impairment and enhancement of endocytic function, respectively. Thus, endocytosis dysfunction may be an underlying cause of ALS pathology.

3.2880 **The small vesicular culprits: the investigation of extracellular vesicles as new targets for cancer treatment**
Urabe, F., Kosaka, N., Yoshioka, Y., Egawa, S. and Ochiya, T.
Clin. Trans. Med., ***6****:45 (2017)*

Extracellular vesicles (EVs) are membranous vesicles released from almost all type of cells including cancer cells. EVs transfer their components, such as microRNAs (miRNAs), messenger RNAs, lipids and proteins, from one cell to another, affecting the target cells. Emerging evidence suggests that reciprocal interactions between cancer cells and the cells in their microenvironment via EVs drive disease progression and therapy resistance. Therefore, understanding the roles of EVs in cancer biology will provide us with new opportunities to treat patients. EVs are also useful for monitoring disease processes. EVs have been found in many kinds of biological fluids such as blood, urine, saliva and semen. Because of their accessibility, EVs offer ease of collection with minimal discomfort to patients and are preferred for serial collection. In addition, they reflect and carry dynamic changes in disease, allowing us to access crucial molecular information about the disease status. Therefore, EVs hold great possibility as clinically useful biomarkers to provide multiple non-invasive snapshots of primary and metastatic tumors. In this review, we summarize current knowledge of miRNAs in EVs in cancer biology and as biomarkers. Furthermore, we discuss the potential of miRNAs in EVs for clinical application.

3.2881 **A method for the isolation and enrichment of purified bovine milk exosomes**
Vaswani, K., Koh, Y.Q., Almughlliq, F.B. and Peiris, H.N.'
Reprod. Biol., ***17****, 341-348 (2017)*

Exosomes are nanovesicles that play important roles in intercellular communication as they carry information to target cells. Isolation of high purity exosomes will aid in studying the exosomal cargo and quantity as well as how cell-specific messages are carried. We describe a new method incorporating size exclusion chromatography (SEC) to enrich milk-derived exosomes from extracellular vesicles (EVs). This involved the initial isolation of EVs from bovine milk via milk processing and ultracentrifugation; followed by a new method to enrich exosomes using SEC. This method was compared to buoyant density gradient centrifugation, a widely used method of enrichment. Exosomes were characterised by particle concentration and size (nanoparticle tracking analysis, NTA), morphology (transmission electron microscopy, TEM), presence of exosomal markers (immunoblotting) and protein concentration (bicinchoninic acid assay, BCA). Proteomic profiles of exosomal fractions were analyzed by mass spectrometry using Information Dependant Acquisition. Milk exosomal fractions were shown to contain exosomal markers flotillin-1 (FLOT-1) and tumor susceptibility gene-101 (TSG-101). The new method produced a higher yield of exosomes compared to buoyant density gradient centrifugation. Pooled exosomal fractions exhibited intact morphology by TEM. The use of SEC confirmed the fractionation of exosomes based on size while minimizing the interference with proteins. Tetraspanins CD9 and CD81 were observed via mass spectrometry in exosomal fractions. This new and efficient method confirmed the signatures for exosomes derived from unpasteurized bovine milk. Purification of exosomes is a foundational technique in the study of biomarkers for pathological conditions and effective drug delivery systems.

3.2882 **An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues**
Corces, M.R. et al
Nature Methods, ***14(10)****, 959-962 (2017)*

We present Omni-ATAC, an improved ATAC-seq protocol for chromatin accessibility profiling that works across multiple applications with substantial improvement of signal-to-background ratio and information content. The Omni-ATAC protocol generates chromatin accessibility profiles from archival frozen tissue samples and 50-μm sections, revealing the activities of disease-associated DNA elements in distinct human brain structures. The Omni-ATAC protocol enables the interrogation of personal regulomes in tissue context and translational studies.

3.2883 Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga
Schatz, D., Rosenwasser, S., Malitsky, S.M., Wolf, S.G., Feldmesser, E. and Vardi, A.
Nature Microbiol., ***2***, *1485-1492 (2017)*

Communication between microorganisms in the marine environment has immense ecological impact by mediating trophic-level interactions and thus determining community structure1. Extracellular vesicles (EVs) are produced by bacteria2,3, archaea4, protists5 and metazoans, and can mediate pathogenicity6 or act as vectors for intercellular communication. However, little is known about the involvement of EVs in microbial interactions in the marine environment7. Here we investigated the signalling role of EVs produced during interactions between the cosmopolitan alga Emiliania huxleyi and its specific virus (EhV, Phycodnaviridae)8, which leads to the demise of these large-scale oceanic blooms9,10. We found that EVs are highly produced during viral infection or when bystander cells are exposed to infochemicals derived from infected cells. These vesicles have a unique lipid composition that differs from that of viruses and their infected host cells, and their cargo is composed of specific small RNAs that are predicted to target sphingolipid metabolism and cell-cycle pathways. EVs can be internalized by E. huxleyi cells, which consequently leads to a faster viral infection dynamic. EVs can also prolong EhV half-life in the extracellular milieu. We propose that EVs are exploited by viruses to sustain efficient infectivity and propagation across E. huxleyi blooms. As these algal blooms have an immense impact on the cycling of carbon and other nutrients11,12, this mode of cell–cell communication may influence the fate of the blooms and, consequently, the composition and flow of nutrients in marine microbial food webs.

3.2884 Genetic variation in glia–neuron signalling modulates ageing rate
Yin, J-A., gao, G., Liu, X-J., Hao, Z-Q., Li, K., kang, X-L., Li, H., Shan, Y-H., Hu, W-L., Li, H-P. and Cai, S-Q.
Nature, ***551***, *198-203 (2017)*

The rate of behavioural decline in the ageing population is remarkably variable among individuals. Despite the considerable interest in studying natural variation in ageing rate to identify factors that control healthy ageing, no such factor has yet been found. Here we report a genetic basis for variation in ageing rates in Caenorhabditis elegans. We find that C. elegans isolates show diverse lifespan and age-related declines in virility, pharyngeal pumping, and locomotion. DNA polymorphisms in a novel peptide-coding gene, named regulatory-gene-for-behavioural-ageing-1 (rgba-1), and the neuropeptide receptor gene npr-28 influence the rate of age-related decline of worm mating behaviour; these two genes might have been subjected to recent selective sweeps. Glia-derived RGBA-1 activates NPR-28 signalling, which acts in serotonergic and dopaminergic neurons to accelerate behavioural deterioration. This signalling involves the SIR-2.1-dependent activation of the mitochondrial unfolded protein response, a pathway that modulates ageing. Thus, natural variation in neuropeptide-mediated glia–neuron signalling modulates the rate of ageing in C. elegans.

3.2885 Multiple marker abundance profiling: combining selected reaction monitoring and data-dependent acquisition for rapid estimation of organelle abundance in subcellular samples
Hooper, C.M., Stevens, T.J., Saukkonen, A., Castleden, I.R., Singh, P., Mann, G.W., Fabre, B., Ito, J., Deery, M.J., Lilley, K.S., Petzold, C.J., Millar, A.H., Heazlewood, J.L. and Parsons, H.T.
Plant J., ***92(6)***, *1202-1217 (2017)*

Measuring changes in protein or organelle abundance in the cell is an essential, but challenging aspect of cell biology. Frequently-used methods for determining organelle abundance typically rely on detection of a very few marker proteins, so are unsatisfactory. *In silico* estimates of protein abundances from publicly available protein spectra can provide useful standard abundance values but contain only data from tissue proteomes, and are not coupled to organelle localization data. A new protein abundance score, the normalized protein abundance scale (NPAS), expands on the number of scored proteins and the scoring accuracy of lower-abundance proteins in Arabidopsis. NPAS was combined with subcellular protein

localization data, facilitating quantitative estimations of organelle abundance during routine experimental procedures. A suite of targeted proteomics markers for subcellular compartment markers was developed, enabling independent verification of *in silico* estimates for relative organelle abundance. Estimation of relative organelle abundance was found to be reproducible and consistent over a range of tissues and growth conditions. *In silico* abundance estimations and localization data have been combined into an online tool, multiple marker abundance profiling, available in the SUBA4 toolbox (http://suba.live).

3.2886 Molecular and cellular reorganization of neural circuits in the human lineage
Sousa, A.M.M. et al
*Science, **358(6366)**, 1027-1032 (2017)*

To better understand the molecular and cellular differences in brain organization between human and nonhuman primates, we performed transcriptome sequencing of 16 regions of adult human, chimpanzee, and macaque brains. Integration with human single-cell transcriptomic data revealed global, regional, and cell-type–specific species expression differences in genes representing distinct functional categories. We validated and further characterized the human specificity of genes enriched in distinct cell types through histological and functional analyses, including rare subpallial-derived interneurons expressing dopamine biosynthesis genes enriched in the human striatum and absent in the nonhuman African ape neocortex. Our integrated analysis of the generated data revealed diverse molecular and cellular features of the phylogenetic reorganization of the human brain across multiple levels, with relevance for brain function and disease.

3.2887 Proceedings of the 2017 ISEV symposium on "HIV, NeuroHIV, drug abuse, & EVs"
Hu, G., Yelamanchili, S., Kashanchi, F., haughey, N., Bond, V.C., Witwer, K., pulliam, l. and Buch, S.
*J. Neurovirol., **23(6)**, 935-940 (2017)*

Despite the success of combination antiretroviral therapy (cART), there is increased prevalence of HIV-associated neurocognitive disorders (HAND) in HIV-1-infected individuals on cART, which poses a major health care challenge. Adding further complexity to this long-term antiretroviral use is the comorbidity with drugs of abuse such as morphine, cocaine, and methamphetamine, which can in turn, exacerbate neurologic and cognitive deficits associated with HAND. Furthermore, HIV proteins, such as the transactivator of transcription (Tat) and the envelope protein (gp120), as well as antiretrovirals themselves can also contribute to the progression of neurodegeneration underlying HAND. In the field of NeuroHIV and drug addiction, EVs hold the potential to serve as biomarkers of cognitive dysfunction, targets of therapy, and as vehicles for therapeutic delivery of agents that can ameliorate disease pathogenesis. Based on the success of a previous Satellite Symposium in 2015 at the ISEV meeting in Washington, experts again expanded on their latest research findings in the field, shedding light on the emerging trends in the field of Extracellular Vesicle (EV) biology in NeuroHIV and drug abuse. The satellite symposium sought to align experts in the fields of NeuroHIV and drug abuse to share their latest insights on the role of EVs in regulating neuroinflammation, neurodegeneration, peripheral immune response, and HIV latency in HIV-infected individuals with or without the comorbidity of drug abuse.

3.2888 Radically truncated MeCP2 rescues Rett syndrome-like neurological defects
Tillotson, R., Selfridge, J., Koerner, M.V., Gadalla, K.K.E., Guy, J., De Soussa, D., Hector, R.D., Cobb, S.R. and Bird, A.
*Nature, **550**, 398-401 (2017)*

Heterozygous mutations in the X-linked MECP2 gene cause the neurological disorder Rett syndrome1. The methyl-CpG-binding protein 2 (MeCP2) protein is an epigenetic reader whose binding to chromatin primarily depends on 5-methylcytosine2,3. Functionally, MeCP2 has been implicated in several cellular processes on the basis of its reported interaction with more than 40 binding partners4, including transcriptional co-repressors (for example, the NCoR/SMRT complex5), transcriptional activators6, RNA7, chromatin remodellers8,9, microRNA-processing proteins10 and splicing factors11. Accordingly, MeCP2 has been cast as a multi-functional hub that integrates diverse processes that are essential in mature neurons12. At odds with the concept of broad functionality, missense mutations that cause Rett syndrome are concentrated in two discrete clusters coinciding with interaction sites for partner macromolecules: the methyl-CpG binding domain13 and the NCoR/SMRT interaction domain5. Here we test the hypothesis that the single dominant function of MeCP2 is to physically connect DNA with the NCoR/SMRT complex, by removing almost all amino-acid sequences except the methyl-CpG binding and NCoR/SMRT interaction domains. We find that mice expressing truncated MeCP2 lacking both the N- and C-terminal

regions (approximately half of the native protein) are phenotypically near-normal; and those expressing a minimal MeCP2 additionally lacking a central domain survive for over one year with only mild symptoms. This minimal protein is able to prevent or reverse neurological symptoms when introduced into MeCP2-deficient mice by genetic activation or virus-mediated delivery to the brain. Thus, despite evolutionary conservation of the entire MeCP2 protein sequence, the DNA and co-repressor binding domains alone are sufficient to avoid Rett syndrome-like defects and may therefore have therapeutic utility.

3.2889 Prion protein inhibits fast axonal transport through a mechanism involving casein kinase 2
Zamponi, E.,Buratti, F., Cataldi, G., Caicedo, H-H., Song, Y., Jungbauer, L.M., LaDu, M.J., Bisbal, M., Lorenzo, A., Ma, J., Helguera, P.R., Morfini, G.A., Brady, S.T. and Pigino, G.F.
PloS One, ***12(12)***, *e0188340 (2017)*

Prion diseases include a number of progressive neuropathies involving conformational changes in cellular prion protein (PrP^c) that may be fatal sporadic, familial or infectious. Pathological evidence indicated that neurons affected in prion diseases follow a dying-back pattern of degeneration. However, specific cellular processes affected by PrP^c that explain such a pattern have not yet been identified. Results from cell biological and pharmacological experiments in isolated squid axoplasm and primary cultured neurons reveal inhibition of fast axonal transport (FAT) as a novel toxic effect elicited by PrP^c. Pharmacological, biochemical and cell biological experiments further indicate this toxic effect involves casein kinase 2 (CK2) activation, providing a molecular basis for the toxic effect of PrP^c on FAT. CK2 was found to phosphorylate and inhibit light chain subunits of the major motor protein conventional kinesin. Collectively, these findings suggest CK2 as a novel therapeutic target to prevent the gradual loss of neuronal connectivity that characterizes prion diseases.

3.2890 Autophagosomal Content Profiling Reveals an LC3C-Dependent Piecemeal Mitophagy Pathway
Le Guerroue, F., Eck, F., Jung, J., Starzetz, T., Mittelbronn, M., Kaulich, M. and Behrends, C.
Mol. Cell, ***68(4)***, *786-796 (2017)*

Autophagy allows the degradation of cytosolic endogenous and exogenous material in the lysosome. Substrates are engulfed by double-membrane vesicles, coined autophagosomes, which subsequently fuse with lysosomes. Depending on the involvement of specific receptor proteins, autophagy occurs in a selective or nonselective manner. While this process is well understood at the level of bulky cargo such as mitochondria and bacteria, we know very little about individual proteins and protein complexes that are engulfed and degraded by autophagy. In contrast to the critical role of autophagy in balancing proteostasis, our current knowledge of the autophagic degradome is very limited. Here, we combined proximity labeling with quantitative proteomics to systematically map the protein inventory of autophagosomes. Using this strategy, we uncovered a basal, housekeeping mitophagy pathway that involves piecemeal degradation of mitochondrial proteins in a LC3C- and p62-dependent manner and contributes to mitochondrial homeostasis maintenance when cells rely on oxidative phosphorylation.

3.2891 A Link between Linearmycin Biosynthesis and Extracellular Vesicle Genesis Connects Specialized Metabolism and Bacterial Membrane Physiology
Hoefler, B.C., Stubbendieck, R.M., Josyula, N.K., Moisan, S.M., Schulze, E.M. and Straight, P.D.
Cell Chem. Biol., ***24(10)***, *1238-1249 (2017)*

Specialized metabolites support bacterial competitive fitness as antibiotics, signals, pigments, and metal scavengers. Little is known about how specialized metabolites are processed and trafficked for their diverse competitive functions. Linearmycins A and B are linear polyketides with antifungal and antibacterial activity but are colony-localized in imaging mass spectrometry of *Streptomyces* sp. Mg1 (*S.* sp. Mg1). To decipher a connection between colony localization and antibiotic activity, we identified the linearmycin gene cluster and investigated linearmycin production and distribution by *S.* sp. Mg1. Our results uncover a large family of variant linearmycins with limited solubility in aqueous solution. We hypothesized that extracellular vesicles may traffic the lipid-like linearmycins. We found that vesicles isolated from culture supernatants contained linearmycins. Surprisingly, abolishing production of linearmycins in *S.* sp. Mg1 also diminished extracellular vesicle production. Our results reveal integration of linearmycin biosynthesis with production of extracellular vesicles, suggesting a deep connection between specialized metabolism and bacterial membrane physiology.

3.2892 A Carbon Nanotube Optical Reporter Maps Endolysosomal Lipid Flux
Jena, P.V., Roxbury, D., Galassi, T.V., Akkari, L., Horoszko, C.P., Iaea, D.B., Budhathoki-Uprety, J., Pipalia, N., Haka, A.S., Harvey, J.D., Mittal, J., Maxfield, F.R., Joyce, J.A. and Heller, D.A.
ACS Nano, ***11***, *10689-10703 (2017)*

Lipid accumulation within the lumen of endolysosomal vesicles is observed in various pathologies including atherosclerosis, liver disease, neurological disorders, lysosomal storage disorders, and cancer. Current methods cannot measure lipid flux specifically within the lysosomal lumen of live cells. We developed an optical reporter, composed of a photoluminescent carbon nanotube of a single chirality, that responds to lipid accumulation *via* modulation of the nanotube's optical band gap. The engineered nanomaterial, composed of short, single-stranded DNA and a single nanotube chirality, localizes exclusively to the lumen of endolysosomal organelles without adversely affecting cell viability or proliferation or organelle morphology, integrity, or function. The emission wavelength of the reporter can be spatially resolved from within the endolysosomal lumen to generate quantitative maps of lipid content in live cells. Endolysosomal lipid accumulation in cell lines, an example of drug-induced phospholipidosis, was observed for multiple drugs in macrophages, and measurements of patient-derived Niemann–Pick type C fibroblasts identified lipid accumulation and phenotypic reversal of this lysosomal storage disease. Single-cell measurements using the reporter discerned subcellular differences in equilibrium lipid content, illuminating significant intracellular heterogeneity among endolysosomal organelles of differentiating bone-marrow-derived monocytes. Single-cell kinetics of lipoprotein-derived cholesterol accumulation within macrophages revealed rates that differed among cells by an order of magnitude. This carbon nanotube optical reporter of endolysosomal lipid content in live cells confers additional capabilities for drug development processes and the investigation of lipid-linked diseases.

3.2893 Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes
Tkach, m., Kowal, j., Zucchetti, A.E., Enserink, l., Jouve, M., Lankar, D., Saitakis, M., Martin-Jaular, l. and Thery, C.
EMBO J., ***36(20)***, *3012-3028 (2017)*

Exosomes, nano-sized secreted extracellular vesicles (EVs), are actively studied for their diagnostic and therapeutic potential. In particular, exosomes secreted by dendritic cells (DCs) have been shown to carry MHC-peptide complexes allowing efficient activation of T lymphocytes, thus displaying potential as promoters of adaptive immune responses. DCs also secrete other types of EVs of different size, subcellular origin and protein composition, whose immune capacities have not been yet compared to those of exosomes. Here, we show that large EVs (lEVs) released by human DCs are as efficient as small EVs (sEVs), including exosomes, to induce $CD4^+$ T-cell activation *in vitro*. When released by immature DCs, however, lEVs and sEVs differ in their capacity to orient T helper (Th) cell responses, the former favouring secretion of Th2 cytokines, whereas the latter promote Th1 cytokine secretion (IFN-γ). Upon DC maturation, however, these functional differences are abolished, and all EVs become able to induce IFN-γ. Our results highlight the need to comprehensively compare the functionalities of EV subtypes in all patho/physiological systems where exosomes are claimed to perform critical roles.

3.2894 Loss of native α-synuclein multimerization by strategically mutating its amphipathic helix causes abnormal vesicle interactions in neuronal cells
Dettmer, U., Ramalingam, N., von Saucken, V.E., Kim, T-E., Newman, A.J., Terry-Kantor, E., Nuber, S., Ericksson, M., Fanning, S., Bartels, T., Lindquist, S., Levy, O.A. and Selkoe, D.
Hum. Mol. Genet., ***26(18)***, *3466-3481 (2017)*

α-Synuclein (αS) forms round cytoplasmic inclusions in Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Evidence suggests a physiological function of αS in vesicle trafficking and release. In contrast to earlier tenets, recent work indicates that αS normally exists in cells in a dynamic equilibrium between monomers and tetramers/multimers. We engineered αS mutants incapable of multimerization, leading to excess monomers at vesicle membranes. By EM, such mutants induced prominent vesicle clustering, leading to round cytoplasmic inclusions. Immunogold labeling revealed abundant αS intimately associated with vesicles of varied size. Fluorescence microscopy with marker proteins showed that the αS-associated vesicles were of diverse endocytic and secretory origin. An αS '3K' mutant (E35K + E46K + E61K) that amplifies the PD/DLB-causing E46K mutation induced αS-rich vesicle clusters resembling the vesicle-rich areas of Lewy bodies, supporting pathogenic relevance. Mechanistically, E46K can increase αS vesicle binding via membrane-induced amphipathic helix formation, and '3K' further enhances this effect. Another engineered αS variant added hydrophobicity to

the hydrophobic half of αS helices, thereby stabilizing αS-membrane interactions. Importantly, substituting charged for uncharged residues within the hydrophobic half of the stabilized helix not only reversed the strong membrane interaction of the multimer-abolishing αS variant but also restored multimerization and prevented the aberrant vesicle interactions. Thus, reversible αS amphipathic helix formation and dynamic multimerization regulate a normal function of αS at vesicles, and abrogating multimers has pathogenic consequences.

3.2895 A plasmid from an Antarctic haloarchaeon uses specialized membrane vesicles to disseminate and infect plasmid-free cells
Erdmann, S., Tschitischko, B., Zhong, L., Raftery, M.-J. and Cavicchioli, R.
*Nature Microbiol., **2**, 1446-1455 (2017)*

The major difference between viruses and plasmids is the mechanism of transferring their genomic information between host cells. Here, we describe the archaeal plasmid pR1SE from an Antarctic species of haloarchaea that transfers via a mechanism similar to a virus. pR1SE encodes proteins that are found in regularly shaped membrane vesicles, and the vesicles enclose the plasmid DNA. The released vesicles are capable of infecting a plasmid-free strain, which then gains the ability to produce plasmid-containing vesicles. pR1SE can integrate and replicate as part of the host genome, resolve out with fragments of host DNA incorporated or portions of the plasmid left behind, form vesicles and transfer to new hosts. The pR1SE mechanism of transfer of DNA could represent the predecessor of a strategy used by viruses to pass on their genomic DNA and fulfil roles in gene exchange, supporting a strong evolutionary connection between plasmids and viruses.

3.2896 Analysis of the small non-protein-coding RNA profile of mouse spermatozoa reveals specific enrichment of piRNAs within mature spermatozoa
Hutcheon, K., McLaughlin, E.A., Stanger, S.J., Bernstein, I.R., Dun, M.D., Eamens, A.L. and Nixon, B.
*RNA Biology, **14(12)**, 1776-2790 (2017)*

Post-testicular sperm maturation and storage within the epididymis is a key determinant of gamete quality and fertilization competence. Here we demonstrate that mouse spermatozoa possess a complex small non-protein-coding RNA (sRNA) profile, the composition of which is markedly influenced by their epididymal transit. Thus, although microRNAs (miRNAs) are highly represented in the spermatozoa of the proximal epididymis, this sRNA class is largely diminished in mature spermatozoa of the distal epididymis. Coincident with this, a substantial enrichment in Piwi-interacting RNA (piRNA) abundance in cauda spermatozoa was detected. Further, features of cauda piRNAs, including; predominantly 29–31 nts in length; preference for uracil at their 5' terminus; no adenine enrichment at piRNA nt 10, and; predominantly mapping to intergenic regions of the mouse genome, indicate that these piRNAs are generated by the PIWIL1-directed primary piRNA production pathway. Accordingly, PIWIL1 was detected via immunoblotting and mass spectrometry in epididymal spermatozoa. These data provide insight into the complexity and dynamic nature of the sRNA profile of spermatozoa and raise the intriguing prospect that piRNAs are generated *in situ* in maturing spermatozoa. Such information is of particular interest in view of the potential role for paternal sRNAs in influencing conception, embryo development and intergenerational inheritance.

3.2897 Exosome secretion promotes chemotaxis of cancer cells
Sung, B.H. and Weaver, A.M.
*Cell Adhesion and Migration, **11(2)**, 187-195 (2017)*

Migration of cells toward chemical cues, or chemotaxis, is important for many biologic processes such as immune defense, wound healing and cancer metastasis. Although chemotaxis is thought to occur in cancer cells, it is less well characterized than chemotaxis of professional immune cells such as neutrophils. Here, we show that cancer cell chemotaxis relies on secretion of exosome-type extracellular vesicles. Migration of fibrosarcoma cells toward a gradient of exosome-depleted serum was diminished by knockdown of the exosome secretion regulator Rab27a. Rescue experiments in which chemotaxis chambers were coated with purified extracellular vesicles demonstrate that exosomes but not microvesicles affect both speed and directionality of migrating cells. Chamber coating with purified fibronectin and fibronectin-depleted exosomes demonstrates that the exosome cargo fibronectin promotes cell speed but cannot account for the role of exosomes in promoting directionality of fibrosarcoma cell movement during chemotaxis. These experiments indicate that exosomes contain multiple motility-promoting cargoes that contribute to different

aspects of cell motility.

3.2898 The isolation of morphologically intact and biologically active extracellular vesicles from the secretome of cancer-associated adipose tissue
Jeurissen, S., Vergauwen, G., Van Deun, J., Lapeire, L., Depoorter, V, Miinalainen, I et al.
*Cell Adhesion & Migration, **11(2)**, 196-204 (2017)*

Breast cancer cells closely interact with different cell types of the surrounding adipose tissue to favor invasive growth and metastasis. Extracellular vesicles (EVs) are nanometer-sized vesicles secreted by different cell types that shuttle proteins and nucleic acids to establish cell-cell communication. To study the role of EVs released by cancer-associated adipose tissue in breast cancer progression and metastasis a standardized EV isolation protocol that obtains pure EVs and maintains their functional characteristics is required. We implemented differential ultracentrifugation as a pre-enrichment step followed by OptiPrep density gradient centrifugation (dUC-ODG) to isolate EVs from the conditioned medium of cancer-associated adipose tissue. A combination of immune-electron microscopy, nanoparticle tracking analysis (NTA) and Western blot analysis identified EVs that are enriched in flotillin-1, CD9 and CD63, and sized between 20 and 200 nm with a density of 1.076–1.125 g/ml. The lack of protein aggregates and cell organelle proteins confirmed the purity of the EV preparations. Next, we evaluated whether dUC-ODG isolated EVs are functionally active. ZR75.1 breast cancer cells treated with cancer-associated adipose tissue-secreted EVs from breast cancer patients showed an increased phosphorylation of CREB. MCF-7 breast cancer cells treated with adipose tissue-derived EVs exhibited a stronger propensity to form cellular aggregates. In conclusion, dUC-ODG purifies EVs from conditioned medium of cancer-associated adipose tissue, and these EVs are morphologically intact and biologically active.

3.2899 Exosomes as secondary inductive signals involved in kidney organogenesis
Krause, M., Rak-Raszewska, A., Naillat, F., Saarela, U., Schmidt, C., Ronkainen, V-P., Bart, G., Ylä-Herttuala, S. and Vainio, S.J.
*J. Extracellular Vesicles, **7(1)**, 1422675 (2018)*

The subfraction of extracellular vesicles, called exosomes, transfers biological molecular information not only between cells but also between tissues and organs as nanolevel signals. Owing to their unique properties such that they contain several RNA species and proteins implicated in kidney development, exosomes are putative candidates to serve as developmental programming units in embryonic induction and tissue interactions. We used the mammalian metanephric kidney and its nephron-forming mesenchyme containing the nephron progenitor/stem cells as a model to investigate if secreted exosomes could serve as a novel type of inductive signal in a process defined as embryonic induction that controls organogenesis. As judged by several characteristic criteria, exosomes were enriched and purified from a cell line derived from embryonic kidney ureteric bud (UB) and from primary embryonic kidney UB cells, respectively. The cargo of the UB-derived exosomes was analysed by qPCR and proteomics. Several miRNA species that play a role in Wnt pathways and enrichment of proteins involved in pathways regulating the organization of the extracellular matrix as well as tissue homeostasis were identified. When labelled with fluorescent dyes, the uptake of the exosomes by metanephric mesenchyme (MM) cells and the transfer of their cargo to the cells can be observed. Closer inspection revealed that besides entering the cytoplasm, the exosomes were competent to also reach the nucleus. Furthermore, fluorescently labelled exosomal RNA enters into the cytoplasm of the MM cells. Exposure of the embryonic kidney-derived exosomes to the whole MM in an *ex vivo* organ culture setting did not lead to an induction of nephrogenesis but had an impact on the overall organization of the tissue. We conclude that the exosomes provide a novel signalling system with an apparent role in secondary embryonic induction regulating organogenesis.

3.2900 Characterization of Trichuris muris secreted proteins and extracellular vesicles provides new insights into host–parasite communication
Eichenberger, R.M., Talukder, M.H., Field, M.A., Wangchuk, P., Giacomin, P., Loukas, A. and Sotillo, J.
*J. Extracellular Vesicles, **7(1)**, 1428004 (2017)*

Whipworms are parasitic nematodes that live in the gut of more than 500 million people worldwide. Owing to the difficulty in obtaining parasite material, the mouse whipworm *Trichuris muris* has been extensively used as a model to study human whipworm infections. These nematodes secrete a multitude of compounds that interact with host tissues where they orchestrate a parasitic existence. Herein we provide the first comprehensive characterization of the excretory/secretory products of *T. muris*. We identify 148 proteins secreted by *T. muris* and show for the first time that the mouse whipworm secretes exosome-like

extracellular vesicles (EVs) that can interact with host cells. We use an Optiprep® gradient to purify the EVs, highlighting the suitability of this method for purifying EVs secreted by a parasitic nematode. We also characterize the proteomic and genomic content of the EVs, identifying >350 proteins, 56 miRNAs (22 novel) and 475 full-length mRNA transcripts mapping to *T. muris* gene models. Many of the miRNAs putatively mapped to mouse genes are involved in regulation of inflammation, implying a role in parasite-driven immunomodulation. In addition, for the first time to our knowledge, colonic organoids have been used to demonstrate the internalization of parasite EVs by host cells. Understanding how parasites interact with their host is crucial to develop new control measures. This first characterization of the proteins and EVs secreted by *T. muris* provides important information on whipworm–host communication and forms the basis for future studies.

3.2901 Salmonella enterica Serovar Typhimurium Alters the Extracellular Proteome of Macrophages and Leads to the Production of Proinflammatory Exosomes
Hui, W.W., Hercik, K., Belsare, S., Alugubelly, N., Clapp, B., Rinaldi, C. and Edelmann, M.J.
Infect. Immun., ***86(2)***, *e00386-17 (2018)*

Salmonella enterica serovar Typhimurium is a Gram-negative bacterium, which can invade and survive within macrophages. Pathogenic salmonellae induce the secretion of specific cytokines from these phagocytic cells and interfere with the host secretory pathways. In this study, we describe the extracellular proteome of human macrophages infected with *S.* Typhimurium, followed by analysis of canonical pathways of proteins isolated from the extracellular milieu. We demonstrate that some of the proteins secreted by macrophages upon *S.* Typhimurium infection are released via exosomes. Moreover, we show that infected macrophages produce $CD63^+$ and $CD9^+$ subpopulations of exosomes at 2 h postinfection. Exosomes derived from infected macrophages trigger the Toll-like receptor 4-dependent release of tumor necrosis factor alpha (TNF-α) from naive macrophages and dendritic cells, but they also stimulate secretion of such cytokines as RANTES, IL-1ra, MIP-2, CXCL1, MCP-1, sICAM-1, GM-CSF, and G-CSF. Proinflammatory effects of exosomes are partially attributed to lipopolysaccharide, which is encapsulated within exosomes. In summary, we show for the first time that proinflammatory exosomes are formed in the early phase of macrophage infection with *S.* Typhimurium and that they can be used to transfer cargo to naive cells, thereby leading to their stimulation.

3.2902 Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer
Halvaei, S., Daryani, S., Eslami-S, Z., Samadi, T., Jafarbeik-Iravani, N., Bakhshayesh, T.O., Majidzadeh-A, K. and Esmaeili, R.
Molecular Therapy – Nucleic Acids, ***10***, *131-141 (2018)*

The important challenge about cancer is diagnosis in primary stages and proper treatment. Although classical clinico-pathological features of the tumor have major prognostic value, the advances in diagnosis and treatment are indebted to discovery of molecular biomarkers and control of cancer in the pre-invasive state. Moreover, the efficiency of available therapeutic options is highly diminished, and chemotherapy is still the main treatment due to lack of enough specific targets. Accordingly, finding the new noninvasive biomarkers for cancer is still an important clinical challenge that is not achieved yet. There are current technologies to screen, diagnose, prognose, and treat cancer, but the limitations of these implements and procedures are undeniable. Liquid biopsy as a noninvasive method has a promising future in the field of cancer, and exosomes as one of the recent areas have drawn much attention. In this review, the potential capability of exosomes is summarized in cancer with the special focus on breast cancer as the second cause of cancer mortality in women all around the world. It discusses reasons to choose exosomes for liquid biopsy and the studies related to different potential biomarkers found in the exosomes. Moreover, exosome studies on milk as a specific biofluid are also discussed. At last, because choosing the method for exosome studies is very challenging, a summary of different techniques is provided.

3.2903 Proteolytic maturation of Drosophila Neuroligin 3 by tumor necrosis factor α-converting enzyme in the nervous system
Wu, J., Tao, Y., Tian, Y., Xing, G., Lv, H., Han, J., Lin, C. and Xie, W.
BBA – General Subjects, ***1862***, *440-450 (2018)*

Background
The functions of autism-associated Neuroligins (Nlgs) are modulated by their post-translational modifications, such as proteolytic cleavage. A previous study has shown that there are different endogenous forms of DNlg3 in *Drosophila*, indicating it may undergo proteolytic processing. However,

the molecular mechanism underlying DNlg3 proteolytic processing is unknown. Here, we report a novel proteolytic mechanism that is essential for DNlg3 maturation and function in the nervous system.
Methods
Molecular cloning, cell culture, immunohistochemistry, western blotting and genetic studies were employed to map the DNlg3 cleavage region, identify the protease and characterize the cleavage manner. Behavior analysis, immunohistochemistry and genetic manipulations were employed to study the functions of different DNlg3 forms in the nervous system and neuromuscular junction (NMJs).
Results
Tumor necrosis factor α-converting enzyme (TACE) cleaved DNlg3 exclusively at its extracellular acetylcholinesterase-like domain to generate the N-terminal fragment and the short membrane-anchored fragment (sDNlg3). DNlg3 was constitutively processed in an activity-independent manner. Interestingly, DNlg3 was cleaved intracellularly in the Golgi apparatus before it arrived at the cell surface, a unique cleavage mechanism that is distinct from 'conventional' ectodomain shedding of membrane proteins, including rodent Nlg1. Genetic studies showed that sDNlg3 was essential for maintaining proper locomotor activity in *Drosophila*.
Conclusions
Our results revealed a unique cleavage mechanism of DNlg3 and a neuron-specific role for DNlg3 maturation which is important in locomotor activity.

3.2904 **Intercellular transfer of pathogenic α-synuclein by extracellular vesicles is induced by the lipid peroxidation product 4-hydroxynonenal**
Zhang, S., Eitan, E., Wu, T-Y. and mattson, M.P.
Neurobiology of Aging, ***61****, 52-65 (2018)*

Parkinson's disease (PD) is characterized by accumulations of toxic α-synuclein aggregates in vulnerable neuronal populations in the brainstem, midbrain, and cerebral cortex. Recent findings suggest that α-synuclein pathology can be propagated transneuronally, but the underlying molecular mechanisms are unknown. Advances in the genetics of rare early-onset familial PD indicate that increased production and/or reduced autophagic clearance of α-synuclein can cause PD. The cause of the most common late-onset PD is unclear, but may involve metabolic compromise and oxidative stress upstream of α-synuclein accumulation. As evidence, the lipid peroxidation product 4-hydroxynonenal (HNE) is elevated in the brain during normal aging and moreso in brain regions afflicted with α-synuclein pathology. Here, we report that HNE increases aggregation of endogenous α-synuclein in primary neurons and triggers the secretion of extracellular vesicles (EVs) containing cytotoxic oligomeric α-synuclein species. EVs released from HNE-treated neurons are internalized by healthy neurons which as a consequence degenerate. Levels of endogenously generated HNE are elevated in cultured cells overexpressing human α-synuclein, and EVs released from those cells are toxic to neurons. The EV-associated α-synuclein is located both inside the vesicles and on their surface, where it plays a role in EV internalization by neurons. On internalization, EVs harboring pathogenic α-synuclein are transported both anterogradely and retrogradely within axons. Focal injection of EVs containing α-synuclein into the striatum of wild-type mice results in spread of synuclein pathology to anatomically connected brain regions. Our findings suggest a scenario for late-onset PD in which lipid peroxidation promotes intracellular accumulation and then extrusion of EVs containing toxic α-synuclein species; the EVs are then internalized by adjacent neurons, so propagating the neurodegenerative process.

3.2905 **Chromosome 19 microRNAs exert antiviral activity independent from type III interferon signaling**
Bayer, A., lennemann, N.J., Ouyang, Y., Sadovsky, E., Sheridan, M.A., Roberts, R.M., Coyne, C.B. and Sadovsky, Y.
Placenta, ***61****, 33-38 (2018)*

Introduction
Cultured primary human trophoblasts (PHT), derived from term placentas, are relatively resistant to infection by diverse viruses. The resistance can be conferred to non-trophoblastic cells by pre-exposing them to medium that was conditioned by PHT cells. This antiviral effect is mediated, at least in part, by microRNAs (miRNA) expressed from the chromosome 19 microRNA cluster (C19MC). Recently we showed that PHT cells and cells pre-exposed to PHT medium are also resistant to infection by Zika virus (ZIKV), an effect mediated by the constitutive release of the type III interferons (IFN) IFN lambda-1 and IFN lambda-2 in trophoblastic medium. We hypothesized that trophoblastic C19MC miRNA are active against ZIKV, and assessed the interaction of this pathway with IFN lambda-1 - mediated resistance.
Methods

Term PHT cells were cultured using standard techniques. An osteosarcoma cell line (U2OS) was used as non-trophoblastic cells, which were infected with either ZIKV or vesicular stomatitis virus (VSV). Trophoblastic extracellular vesicles (EVs) were produced by gradient ultracentrifugation. RT-qPCR was used to determine viral infection, cellular or medium miRNA levels and the expression of interferon-stimulated genes.
Results
We showed that C19MC miRNA attenuate infection of U2OS cells by ZIKV, and that C19MC miRNA or exosomes that contain C19MC miRNA did not influence the type III IFN pathway. Similarly, cell exposure to recombinant IFN lambda-1 had no effect on miRNA expression, and these pathways did not exhibit synergistic interaction.
Discussion
PHT cells exert antiviral activity by at least two independent mechanisms, mediated by C19MC miRNA and by type III IFNs.

3.2906 **DCF1 subcellular localization and its function in mitochondria**
Chen, Y., Feng, R., Luo, G., Guo, J., Wang, Y., Sun, Y., Zheng, L. and Wen, T.
Biochemie, ***144***, *50-55 (2018)*

Dendritic cell factor 1 (DCF1) is a transmembrane protein that plays important roles in regulating neural stem cell differentiation and dendritic spine formation. Apart from its cytoplasmic functions, DCF1 plays a role in autophagy during the regulation of amyloid precursor proteins. However, the subcellular localization of DCF1 remains unknown. Therefore, in this study, DCF1 tagged with green fluorescent protein was transiently expression in HelaS3 and HEK293T cells. The results showed that DCF1 was widely expressed in different organelles, including the mitochondria, Golgi apparatus, endoplasmic reticulum, endosomes and lysosomes. An iodixanol step gradient further confirmed that DCF1 is localized to the mitochondria, endosomes, lysosomes, endoplasmic reticulum, and proteasome. Finally, functional analysis of the mitochondria revealed that DCF1 affected the expression and localization of MGST1. This study presents a comprehensive evaluation of the subcellular localization of DCF1, which provides important information on complex functions mediated by DCF1.

3.2907 **Evaluation of a method to measure HHV-6B infection in vitro based on cell size**
Becerra-Artiles, A., Santoro, T. and Stern, L.J.
Virology J., ***15***:*4 (2018)*

Background
Human herpesvirus 6 (HHV-6A and HHV-6B) infection of cell cultures can be measured by different methods, including immunofluorescence microscopy, flow cytometry, or quantification of virus DNA by qPCR. These methods are reliable and sensitive but require long processing times and can be costly. Another method used in the field relies on the identification of enlarged cells in the culture; this method requires little sample processing and is relatively fast. However, visual inspection of cell cultures can be subjective and it can be difficult to establish clear criteria to decide if a cell is enlarged. To overcome these issues, we explored a method to monitor HHV-6B infections based on the systematic and objective measurement of the size of cells using an imaging-based automated cell counter.
Results
The size of cells in non-infected and HHV-6B-infected cultures was measured at different times post-infection. The relatively narrow size distribution observed for non-infected cultures contrasted with the broader distributions observed in infected cultures. The average size of cultures shifted towards higher values after infection, and the differences were significant for cultures infected with relatively high doses of virus and/or screened at longer times post-infection. Correlation analysis showed that the trend observed for average size was similar to the trend observed for two other methods to measure infection: amount of virus DNA in supernatant and the percentage of cells expressing a viral antigen. In order to determine the performance of the size-based method in differentiating non-infected and infected cells, receiver operating characteristic (ROC) curves were used to analyze the data. Analysis using size of individual cells showed a moderate performance in detecting infected cells (area under the curve (AUC) ~ 0.80-0.87), while analysis using the average size of cells showed a very good performance in detecting infected cultures (AUC ~ 0.99).
Conclusions
The size-based method proved to be useful in monitoring HHV-6B infections for cultures where a substantial fraction of cells were infected and when monitored at longer times post-infection, with the

advantage of being relatively fast and easy. It is a convenient method for monitoring virus production in-vitro and bulk infection of cells.

3.2908 Glycolysis promotes caspase-3 activation in lipid rafts in T cells
Secinaro, M.A., Fortner, K.A., Dienz, O., Logan, A., Murphy, M.P., Anathy, V., Boyson, J.E. and Budd, R.C.
*Cell Death & Disease, **9**:62 (2018)*

Resting T cells undergo a rapid metabolic shift to glycolysis upon activation in the presence of interleukin (IL)-2, in contrast to oxidative mitochondrial respiration with IL-15. Paralleling these different metabolic states are striking differences in susceptibility to restimulation-induced cell death (RICD); glycolytic effector T cells are highly sensitive to RICD, whereas non-glycolytic T cells are resistant. It is unclear whether the metabolic state of a T cell is linked to its susceptibility to RICD. Our findings reveal that IL-2-driven glycolysis promotes caspase-3 activity and increases sensitivity to RICD. Neither caspase-7, caspase-8, nor caspase-9 activity is affected by these metabolic differences. Inhibition of glycolysis with 2-deoxyglucose reduces caspase-3 activity as well as sensitivity to RICD. By contrast, IL-15-driven oxidative phosphorylation actively inhibits caspase-3 activity through its glutathionylation. We further observe active caspase-3 in the lipid rafts of glycolytic but not non-glycolytic T cells, suggesting a proximity-induced model of self-activation. Finally, we observe that effector T cells during influenza infection manifest higher levels of active caspase-3 than naive T cells. Collectively, our findings demonstrate that glycolysis drives caspase-3 activity and susceptibility to cell death in effector T cells independently of upstream caspases. Linking metabolism, caspase-3 activity, and cell death provides an intrinsic mechanism for T cells to limit the duration of effector function.

3.2909 Current knowledge on exosome biogenesis and release
Hessvik, N.P. and Llorente, A.
*Cell. Mol. Life Sci., **75(2)**, 193-208 (2018)*

Exosomes are nanosized membrane vesicles released by fusion of an organelle of the endocytic pathway, the multivesicular body, with the plasma membrane. This process was discovered more than 30 years ago, and during these years, exosomes have gone from being considered as cellular waste disposal to mediate a novel mechanism of cell-to-cell communication. The exponential interest in exosomes experienced during recent years is due to their important roles in health and disease and to their potential clinical application in therapy and diagnosis. However, important aspects of the biology of exosomes remain unknown. To explore the use of exosomes in the clinic, it is essential that the basic molecular mechanisms behind the transport and function of these vesicles are better understood. We have here summarized what is presently known about how exosomes are formed and released by cells. Moreover, other cellular processes related to exosome biogenesis and release, such as autophagy and lysosomal exocytosis are presented. Finally, methodological aspects related to exosome release studies are discussed.

3.2910 Type A viral hepatitis: A summary and update on the molecular virology, epidemiology, pathogenesis and prevention
Lemon, S.M., Ott, J., Van Damme, P. and Shouval, D.
*J. Hepatol., **68(1)**, 167-184 (2018)*

Although epidemic jaundice was well known to physicians of antiquity, it is only in recent years that medical science has begun to unravel the origins of hepatitis A virus (HAV) and the unique pathobiology underlying acute hepatitis A in humans. Improvements in sanitation and the successful development of highly efficacious vaccines have markedly reduced the worldwide occurence of this enterically-transmitted infection over the past quarter century, yet the virus persists in vulnerable populations and those without HAV immunity and remains a common cause of food-borne disease outbreaks in economically-advantaged societies. Reductions in HAV incidence have led to increases in the median age at which infection occurs, often resulting in more severe disease in affected persons and paradoxical increases in disease burden in some developing nations. Here, we summarize recent advances in the molecular virology and epidemiology of HAV, an atypical member of the *Picornaviridae* family, survey what is known of the pathogenesis of hepatitis A in humans and the host-pathogen interactions that typify the infection. The article also reviews medical and public health aspects of HAV vaccination and disease prevention.

3.2911 **Exosomes serve as novel modes of tick-borne flavivirus transmission from arthropod to human cells and facilitates dissemination of viral RNA and proteins to the vertebrate neuronal cells**
Zhou, W., Woodson, m., Neupane, B., bai, F., Sherman, M.B., Choi, K.H., Neelakanta, G. and Sultana, H.
*PloS Pathogens, **14(1)**, e1006764 (2018)*

Molecular determinants and mechanisms of arthropod-borne flavivirus transmission to the vertebrate host are poorly understood. In this study, we show for the first time that a cell line from medically important arthropods, such as ticks, secretes extracellular vesicles (EVs) including exosomes that mediate transmission of flavivirus RNA and proteins to the human cells. Our study shows that tick-borne Langat virus (LGTV), a model pathogen closely related to tick-borne encephalitis virus (TBEV), profusely uses arthropod exosomes for transmission of viral RNA and proteins to the human- skin keratinocytes and blood endothelial cells. Cryo-electron microscopy showed the presence of purified arthropod/neuronal exosomes with the size range of 30 to 200 nm in diameter. Both positive and negative strands of LGTV RNA and viral envelope-protein were detected inside exosomes derived from arthropod, murine and human cells. Detection of Nonstructural 1 (NS1) protein in arthropod and neuronal exosomes further suggested that exosomes contain viral proteins. Viral RNA and proteins in exosomes derived from tick and mammalian cells were secured, highly infectious and replicative in all tested evaluations. Treatment with GW4869, a selective inhibitor that blocks exosome release affected LGTV loads in both arthropod and mammalian cell-derived exosomes. Transwell-migration assays showed that exosomes derived from infected-brain-microvascular endothelial cells (that constitute the blood-brain barrier) facilitated LGTV RNA and protein transmission, crossing of the barriers and infection of neuronal cells. Neuronal infection showed abundant loads of both tick-borne LGTV and mosquito-borne West Nile virus RNA in exosomes. Our data also suggest that exosome-mediated LGTV viral transmission is clathrin-dependent. Collectively, our results suggest that flaviviruses uses arthropod-derived exosomes as a novel means for viral RNA and protein transmission from the vector, and the vertebrate exosomes for dissemination within the host that may subsequently allow neuroinvasion and neuropathogenesis.

3.2912 **Neuronal lysosomal dysfunction releases exosomes harboring APP C-terminal fragments and unique lipid signatures**
Miranda, A.M., Lasiecka, Z.M., Xu, Y., Neufeld, J., Shahriar, S., Simoes, S., Chan, R.B., Oliveira, T.G., Small, S.A. and Di Paolo, G.
*Nature Communications, **9**:291 (2018)*

Defects in endolysosomal and autophagic functions are increasingly viewed as key pathological features of neurodegenerative disorders. A master regulator of these functions is phosphatidylinositol-3-phosphate (PI3P), a phospholipid synthesized primarily by class III PI 3-kinase Vps34. Here we report that disruption of neuronal Vps34 function in vitro and in vivo impairs autophagy, lysosomal degradation as well as lipid metabolism, causing endolysosomal membrane damage. PI3P deficiency also promotes secretion of unique exosomes enriched for undigested lysosomal substrates, including amyloid precursor protein C-terminal fragments (APP-CTFs), specific sphingolipids, and the phospholipid bis(monoacylglycero)phosphate (BMP), which normally resides in the internal vesicles of endolysosomes. Secretion of these exosomes requires neutral sphingomyelinase 2 and sphingolipid synthesis. Our results reveal a homeostatic response counteracting lysosomal dysfunction via secretion of atypical exosomes eliminating lysosomal waste and define exosomal APP-CTFs and BMP as candidate biomarkers for endolysosomal dysfunction associated with neurodegenerative disorders.

3.2913 **Exosome-Mimetic Nanovesicles from Hepatocytes promote hepatocyte proliferation in vitro and liver regeneration in vivo**
Wu, J-Y., Ji, A-L., Wang, Z-x., Qiang, G-H., Qu, Z., Wu, J-H. and Jiang, C-P.
*Science Reports, **8**:2471 (2018)*

The liver has great regenerative capacity after functional mass loss caused by injury or disease. Many studies have shown that primary hepatocyte-derived exosomes, which can deliver biological information between cells, promote the regenerative process of the liver. However, the yield of exosomes is very limited. Recent studies have demonstrated that exosome-mimetic nanovesicles (NVs) can be prepared from cells with almost 100 times the production yield compared with exosomes. Thus, this study investigated the therapeutic capacity of exosome-mimetic NVs from primary hepatocytes in liver regeneration. Exosome-mimetic NVs were prepared by serial extrusions of cells through polycarbonate membranes, and the yield of these NVs was more than 100 times that of exosomes. The data indicated that the NVs could promote hepatocyte proliferation and liver regeneration by significantly enhancing the content of

sphingosine kinase 2 in recipient cells. To the best of our knowledge, this is the first time that exosome-mimetic NVs from primary hepatocytes have been prepared, and these NVs have components similar to exosomes from primary hepatocytes and, in some respects, biofunctions similar to exosomes. Strategies inspired by this study may lead to substitution of exosomes with exosome-mimetic NVs for biofunctional purposes, including utilization in tissue repair and regeneration.

3.2914 **Acidic organelles mediate TGF-β1-induced cellular fibrosis via (pro)renin receptor and vacuolar ATPase trafficking in human peritoneal mesothelial cells**
Oba-yabana, I., Mori, T., Takahashi, C., Hirose, T., Ohsaki, Y., Kinugasa, S., Muroya, Y., Sato, E., Nguyen, G., Piedagnel, R., Ronco, P.M., Totsune, K. and Ito, S.
*Scientific Reports, **8**:2648 (2018)*

TGF-β1, which can cause renal tubular injury through a vacuolar-type H^+-ATPase (V-ATPase)-mediated pathway, is induced by the glucose degradation product methylglyoxal to yield peritoneal injury and fibrosis. The present study investigated the roles of V-ATPase and its accessory protein, the (pro)renin receptor, in peritoneal fibrosis during peritoneal dialysis. Rats daily administered 20 mM methylglyoxal intraperitoneally developed significant peritoneal fibrosis after 7 days with increased expression of TGF-β and V-ATPase, which was reduced by the inhibition of V-ATPase with co-administration of 100 mM bafilomycin A1. The (pro)renin receptor and V-ATPase were expressed in acidic organelles and cell membranes of human peritoneal mesothelial cells. TGF-β1 upregulated the expression of collagens, α-SMA, and EDA-fibronectin, together with ERK1/2 phosphorylation, which was reduced by inhibition of V-ATPase, (pro)renin receptor, or the MAPK pathway. Fibronectin and the soluble (pro)renin receptor were excreted from cells by acidic organelle trafficking in response to TGF-β1; this excretion was also suppressed by inhibition of V-ATPase. Soluble (pro)renin receptor concentrations in effluents of patients undergoing peritoneal dialysis were associated with the dialysate-to-plasma ratio of creatinine. Together, these results demonstrate a novel fibrosis mechanism through the (pro)renin receptor and V-ATPase in the acidic organelles of peritoneal mesothelial cells.

3.2915 **Optical and surface plasmonic approaches to characterize extracellular vesicles. A review**
Shpacovitch, V. and Hergenröder, R.
*Anal. Chim. Acta, **1005**, 1-15 (2018)*

Extracellular vesicles (EVs) have been recognized as messengers delivering various active molecules between cells. This feature of EVs drew the attention of clinicians as well as researchers from different fields. However, exciting ideas to employ EVs as means of drug delivery or to test them as biomarkers of cellular status require very thoughtful and attentive approaches to the selection of analytical techniques for EV characterization. Optical and surface plasmonic analytical methods offer a researcher an invaluable opportunity to use already sized and/or quantified EVs in further functional cell-based assays and in focused biochemical tests (nucleic acid and protein arrays, etc.). Moreover, a high sensitivity and relative flexibility of surface plasmonic sensors open a possibility to develop instruments performing quantitative, metrical and EV surface/content analysis in a single device. This review aims to consider the applicability of established and modern optical techniques as well as novel surface plasmonic approaches for different aspects of EV analysis.

3.2916 **Tetraspanin CD63 Bridges Autophagic and Endosomal Processes To Regulate Exosomal Secretion and Intracellular Signaling of Epstein-Barr Virus LMP1**
Hurwitz, S.N., Cheerathodi, M.R., Nkosi, D., York, S.B. and Meckes Jr., D.G.
*J. Virol., **92(5)**, e1969-17 (2018)*

The tetraspanin protein CD63 has been recently described as a key factor in extracellular vesicle (EV) production and endosomal cargo sorting. In the context of Epstein-Barr virus (EBV) infection, CD63 is required for the efficient packaging of the major viral oncoprotein latent membrane protein 1 (LMP1) into exosomes and other EV populations and acts as a negative regulator of LMP1 intracellular signaling. Accumulating evidence has also pointed to intersections of the endosomal and autophagy pathways in maintaining cellular secretory processes and as sites for viral assembly and replication. Indeed, LMP1 can activate the mammalian target of rapamycin (mTOR) pathway to suppress host cell autophagy and facilitate cell growth and proliferation. Despite the growing recognition of cross talk between endosomes and autophagosomes and its relevance to viral infection, little is understood about the molecular mechanisms governing endosomal and autophagy convergence. Here, we demonstrate that CD63-dependent vesicle protein secretion directly opposes intracellular signaling activation downstream of

LMP1, including mTOR-associated proteins. Conversely, disruption of normal autolysosomal processes increases LMP1 secretion and dampens signal transduction by the viral protein. Increases in mTOR activation following CD63 knockout are coincident with the development of serum-dependent autophagic vacuoles that are acidified in the presence of high LMP1 levels. Altogether, these findings suggest a key role of CD63 in regulating the interactions between endosomal and autophagy processes and limiting cellular signaling activity in both noninfected and virally infected cells.

3.2917 ***Legionella* effector AnkX interacts with host nuclear protein PLEKHN1**
Yu, X., Noll, R.R., Duenas, B.P., Allgood, S.C., barker, K., Caplan, J.L., machner, M.P., LaBaer, J., Qiu, J. and Neunuebel, M.R.
BMC Microbiol., ***18****:5 (2018)*

Background
The intracellular bacterial pathogen *Legionella pneumophila* proliferates in human alveolar macrophages, resulting in a severe pneumonia termed Legionnaires' disease. Throughout the course of infection, *L. pneumophila* remains enclosed in a specialized membrane compartment that evades fusion with lysosomes. The pathogen delivers over 300 effector proteins into the host cell, altering host pathways in a manner that sets the stage for efficient pathogen replication. The *L. pneumophila* effector protein AnkX targets host Rab GTPases and functions in preventing fusion of the *Legionella*-containing vacuole with lysosomes. However, the current understanding of AnkX's interaction with host proteins and the means through which it exerts its cellular function is limited.
Results
Here, we investigated the protein interaction network of AnkX by using the nucleic acid programmable protein array (NAPPA), a high-density platform comprising 10,000 unique human ORFs. This approach facilitated the discovery of PLEKHN1 as a novel interaction partner of AnkX. We confirmed this interaction through multiple independent in vitro pull-down, co-immunoprecipitation, and cell-based assays. Structured illumination microscopy revealed that endogenous PLEKHN1 is found in the nucleus and on vesicular compartments, whereas ectopically produced AnkX co-localized with lipid rafts at the plasma membrane. In mammalian cells, HaloTag-AnkX co-localized with endogenous PLEKHN1 on vesicular compartments. A central fragment of AnkX (amino acids 491–809), containing eight ankyrin repeats, extensively co-localized with endogenous PLEKHN1, indicating that this region may harbor a new function. Further, we found that PLEKHN1 associated with multiple proteins involved in the inflammatory response.
Conclusions
Altogether, our study provides evidence that in addition to Rab GTPases, the *L. pneumophila* effector AnkX targets nuclear host proteins and suggests that AnkX may have novel functions related to manipulating the inflammatory response.

3.2918 **Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer**
Bhome, R., Del Vecchio, F., Lee, G-H., Buillock, M.D., Primrose, J.N., Sayan, A.E. and Mirnezami, A.H.
Cancer Lett., ***420****, 228-235 (2018)*

Exosomes are secreted vesicles which can transmit molecular cargo between cells. Exosomal microRNAs (exomiRs) have drawn much attention in recent years because there is increasing evidence to suggest that loading of microRNAs into exosomes is not a random process. Preclinical studies have identified functional roles for exomiRs in influencing many hallmarks of cancer. Mechanisms underpinning their actions, such as exomiR receptors ("miRceptors"), are now becoming apparent. Even more exciting is the fact that exomiRs are highly suitable candidates for use as non-invasive biomarkers in an era of personalized cancer medicine.

3.2919 **Preparation of Highly Enriched ER Membranes Using Free-Flow Electrophoresis**
Parsons, H.T.
Methods in Mol. Biol., ***1691****, 103-115 (2018)*

Free-flow electrophoresis (FFE) is a technique for separation of proteins, peptides, organelles, and cells. With zone electrophoresis (ZE-FFE), organelles are separated according to surface charge. The ER is the only remaining major cellular compartment in Arabidopsis not to have been isolated using density centrifugation, immune-isolation, or any other method previously applied to purification of plant membranes. By using continuous-flow electrophoresis ER vesicles of similar surface charge, which may have been fragmented during cell lysis, can be focused. A large portion of these vesicles are of sufficiently

different surface charge that separation from the majority of Golgi and other contaminants is possible. Here we adapt an earlier ZE-FFE Golgi isolation protocol for the isolation of highly pure ER vesicles and for tracking the migration of peripheral ER vesicles. Isolating ER vesicles of homogenous surface charge allows multi-'omic analyses to be performed on the ER. This facilitates investigations into structure–function relationships within the ER.

3.2920 Methods to Enrich Exosomes from Conditioned Media and Biological Fluids
Sharma, S., Scholz-Romero, K., Rice, G.E. and Salomon, C.
*Methods in Mol. Biol., **1710**, 103-115 (2018)*

Exosomes are nano-vesicles which can transport a range of molecules including but not limited to proteins and miRNA. This ability of exosomes renders them useful in cellular communication often resulting in biological changes. They have several functions in facilitating normal biological processes such as immune responses and an involvement in pregnancy. However, they have also been linked to pathological conditions including cancer and pregnancy complications such as preeclampsia. An understanding for the role of exosomes in preeclampsia is based on the ability to purify and characterize exosomes. There have been several techniques proposed for the enrichment of exosomes such as ultracentrifugation, density gradient separation, and ultrafiltration although there is no widely accepted optimized technique. Here we describe a workflow for isolating exosomes from cell-conditioned media and biological fluids using a combination of centrifugation, buoyant density, and ultrafiltration approaches.

3.2921 Overview of Protocols for Studying Extracellular RNA and Extracellular Vesicles
Small, J., Alexander, R. and Balaj, L.
*Methods in Mol. Biol., **1740**, 17-21 (2018)*

Understanding the role of extracellular RNA (exRNA) has emerged as an exciting avenue for biomarker, therapeutic, as well as basic cell–cell communication applications and discoveries. Multiple protocols, kits, and procedures have been developed in the last decade to allow fractionation as well as isolation of subpopulations of macromolecules of interest found in biofluids. Here, we introduce the protocols decision tree developed by the Extracellular RNA Communication Consortium and available on their website (exRNA portal), and compare all methods currently available to the exRNA field and report pros and cons for each platform.

3.2922 Extracellular RNA Isolation from Cell Culture Supernatant
Bakr, S., Simonson, B., Danielson, K.M. and Das, S.
*Methods in Mol. Biol., **1740**, 23-34 (2018)*

Extracellular RNAs are emerging as novel biomarkers and mediators of intercellular communication. Various methods to isolate RNA from biofluids and cell culture supernatants have been previously used by investigators. Here, we describe several standardized protocols for the isolation of RNAs from cell culture supernatants that utilize commercially available kits and reagents.

3.2923 Cushioned–Density Gradient Ultracentrifugation (C-DGUC): A Refined and High Performance Method for the Isolation, Characterization, and Use of Exosomes
Li, K., Wong, D.K., Hong, K-Y. and Raffia, R.L.
Methods in Mol. Biol., 1740, 69-83 (2018)

Exosomes represent one class of extracellular vesicles that are thought to be shed by all cell types. Although the exact nature of exosome biogenesis and function remains incompletely understood, they are increasingly recognized as a source of intercellular communication in health and disease. Recent observations of RNA exchange via donor cell-derived exosomes that exert genetic regulation in recipient cells have led to a boon into exosome research. The excitement and promise of exosomes as a new therapeutic avenue for human pathologies remain limited by challenges associated with their isolation from culture media and biofluids. The introduction of new methodologies to facilitate the isolation of exosomes has simultaneously raised concerns related to the reproducibility of studies describing exosome effector functions. Even high-speed ultracentrifugation, the first and long considered gold standard approach for exosome isolation has recently been noted to be subject to uncontrolled variables that could impact functional readouts of exosome preparations. This chapter describes principles and methods that attempt to overcome such limitations by first concentrating exosomes in a liquid cushion and subsequently resolving them using density gradient ultracentrifugation. Our approach avoids possible complications associated

with direct pelleting onto plastic tubes and allows for further purification of exosomes from dense protein aggregates.

3.2924 Dimerization leads to changes in APP (amyloid precursor protein) trafficking mediated by LRP1 and SorLA

Eggert, S., Gonzalez, A.C., Thomas, C., Schilling, S., Schwarz, S.M., Tischer, C., Adam, V., Strecker, P., Schmidt, V., Willnow, T.E., Hermey, G., Pietrzik, C.U., Koo, E.H. and Kins, S.
Cell Mol. Life Sci., ***75***, *301.322 (2018)*

Proteolytic cleavage of the amyloid precursor protein (APP) by α-, β- and γ-secretases is a determining factor in Alzheimer's disease (AD). Imbalances in the activity of all three enzymes can result in alterations towards pathogenic Aβ production. Proteolysis of APP is strongly linked to its subcellular localization as the secretases involved are distributed in different cellular compartments. APP has been shown to dimerize in *cis*-orientation, affecting Aβ production. This might be explained by different substrate properties defined by the APP oligomerization state or alternatively by altered APP monomer/dimer localization. We investigated the latter hypothesis using two different APP dimerization systems in HeLa cells. Dimerization caused a decreased localization of APP to the Golgi and at the plasma membrane, whereas the levels in the ER and in endosomes were increased. Furthermore, we observed via live cell imaging and biochemical analyses that APP dimerization affects its interaction with LRP1 and SorLA, suggesting that APP dimerization modulates its interplay with sorting molecules and in turn its localization and processing. Thus, pharmacological approaches targeting APP oligomerization properties might open novel strategies for treatment of AD.

3.2925 Lipid composition of membrane microdomains isolated detergent-free from PUFA supplemented RAW264.7 macrophages

Hellwwing, C., Tigistu-Sahle, F., Fuhrmann, H., Käkelä, R. and Schumann, J.
J. Cell Physiol., ***233(3)***, *2602-2612 (2018)*

Profound alterations in the lipid profile of raft and non-raft plasma membrane microdomains were found when RAW264.7 macrophages were supplemented with polyunsaturated fatty acids (PUFAs) in physiologically relevant concentrations. For the first time lipids in the detergent-free isolated membrane domains of phagocytic immune cells were characterized by mass spectrometry. The extent of remodeling of the membrane lipids differed with different n3 and n6 PUFA supplements. The mildest effects were detected for α-linolenic acid (LNA) and linoleic acid (LA), the C18 precursors of the n3 and n6 families, respectively. When the effects of highly unsaturated PUFAs were compared, eicosapentaenoic acid (EPA) caused more extensive restructuring of membrane lipids than docosahexaenoic acid (DHA) or arachidonic acid (AA). The supplements altered the lipid species composition of both the raft and non-raft membrane fractions. The rafts containing elevated proportions of highly unsaturated lipid species may relocate sterically incompatible lipids and proteins originally belonging to this microdomain. Such effect was evident for sphingomyelin, which favored non-rafts instead of rafts after EPA supplementation. The current work suggests that the different functional consequences found previously when supplementing macrophages with either EPA or DHA have their origin in the different effects of these PUFAs on membrane architecture.

3.2926 TMD1 domain and CRAC motif determine the association and disassociation of MxIRT1 with detergent-resistant membranes

Tan, S., Zhang, P., Xiao, W., Feng, B., Chen, L-Y., Li, S., Li, P., Zhao, W-Z., Qi, X-T. and Yin, L-P.
Traffic, ***19(2)***, *122-137 (2018)*

Iron is essential for most living organisms. The iron-regulated transporter1 (IRT1) plays a major role in iron uptake in roots, and its trafficking from endoplasmic reticulum (ER) to plasma membrane (PM) is tightly coordinated with changes in iron environment. However, studies on the IRT1 response are limited. Here, we report that *Malus xiaojinesis* IRT1 (MxIRT1) associates with detergent-resistant membranes (DRMs, a biochemical counterpart of PM microdomains), whereas the PM microdomains are known platforms for signal transduction in the PM. Depending on the shift of MxIRT1 from microdomains to homogeneous regions in PM, MxIRT1-mediated iron absorption is activated by the cholesterol recognition/interaction amino acid consensus (CRAC) motif of MxIRT1. MxIRT1 initially associates with DRMs in ER via its transmembrane domain 1 (TMD1), and thus begins DRMs-dependent intracellular trafficking. Subsequently, MxIRT1 is sequestered in COPII vesicles via the ER export signal sequence in

MxIRT1. These studies suggest that iron homeostasis is influenced by the CRAC motif and TMD1 domain due to their determination of MxIRT1-DRMs association.

3.2927 **The hydrophobic region of the Leishmania peroxin 14: requirements for association with a glycosome mimetic membrane**
Cyr, N., Smith, T.K., Boisselier, E., Leroux, L-P., Kottarampatel, A.H., Davidsen, A., Salesse, C. and Jardim, A.
Biochem. J., ***475***, *511-529 (2018)*

Protein import into the *Leishmania* glycosome requires docking of the cargo-loaded peroxin 5 (PEX5) receptor to the peroxin 14 (PEX14) bound to the glycosome surface. To examine the LdPEX14–membrane interaction, we purified *L. donovani* promastigote glycosomes and determined the phospholipid and fatty acid composition. These membranes contained predominately phosphatidylethanolamine, phosphatidylcholine, and phosphatidylglycerol (PG) modified primarily with C18 and C22 unsaturated fatty acid. Using large unilamellar vesicles (LUVs) with a lipid composition mimicking the glycosomal membrane in combination with sucrose density centrifugation and fluorescence-activated cell sorting technique, we established that the LdPEX14 membrane-binding activity was dependent on a predicted transmembrane helix found within residues 149–179. Monolayer experiments showed that the incorporation of PG and phospholipids with unsaturated fatty acids, which increase membrane fluidity and favor a liquid expanded phase, facilitated the penetration of LdPEX14 into biological membranes. Moreover, we demonstrated that the binding of LdPEX5 receptor or LdPEX5–PTS1 receptor–cargo complex was contingent on the presence of LdPEX14 at the surface of LUVs.

3.2928 **The MTM1–UBQLN2–HSP complex mediates degradation of misfolded intermediate filaments in skeletal muscle**
Gavrilidis, C., Laredj, L., Solinhac, R., Messaddeq, n., Viaud, J., laporte, J., Sumara, I. and Hnia, K.
Nature Cell Biol., ***20***, *198-210 (2018)*

The ubiquitin proteasome system and autophagy are major protein turnover mechanisms in muscle cells, which ensure stemness and muscle fibre maintenance. Muscle cells contain a high proportion of cytoskeletal proteins, which are prone to misfolding and aggregation; pathological processes that are observed in several neuromuscular diseases called proteinopathies. Despite advances in deciphering the mechanisms underlying misfolding and aggregation, little is known about how muscle cells manage cytoskeletal degradation. Here, we describe a process by which muscle cells degrade the misfolded intermediate filament proteins desmin and vimentin by the proteasome. This relies on the MTM1–UBQLN2 complex to recognize and guide these misfolded proteins to the proteasome and occurs prior to aggregate formation. Thus, our data highlight a safeguarding function of the MTM1–UBQLN2 complex that ensures cytoskeletal integrity to avoid proteotoxic aggregate formation.

3.2929 **HIV Activates the Tyrosine Kinase Hck to Secrete ADAM Protease-Containing Extracellular Vesicles**
Lee, J-H., Ostalecki, C., Zhao, Z., Kesti, T., Bruns, H., Simon, B., Harrer, T., Saksela, K. and Baur, A.S.
EBioMed., ***28***, *151-161 (2018)*

HIV-Nef activates the myeloid cell-typical tyrosine kinase Hck, but its molecular role in the viral life cycle is not entirely understood. We found that HIV plasma extracellular vesicles (HIV pEV) containing/10 proteases and Nef also harbor Hck, and analyzed its role in the context of HIV pEV secretion. Myeloid cells required Hck for the vesicle-associated release of ADAM17. This could be induced by the introduction of Nef and implied that HIV targeted Hck for vesicle-associated ADAM17 secretion from a myeloid compartment. The other contents of HIV-pEV, however, including miRNA and effector protein profiles, as well as the presence of haptoglobin suggested hepatocytes as a possible cellular source. HIV liver tissue analysis supported this assumption, revealing induction of Hck translation, evidence for ADAM protease activation and HIV infection. Our findings suggest that HIV targets Hck to induce pro-inflammatory vesicles release and identifies hepatocytes as a possible host cell compartment.

3.2930 **Exosome and MiRNA in Stroke**
Bihl, J., Wang, J., Ma, X., Yang, Y., Zhao, B. and Chen, Y.
Cellular and Molecular Approaches to Regeneration and Repair (Springer), 325-361 (2018)

Stroke is one of the leading causes of death and disability worldwide. Various types of stem cells have been applied to treat stroke and have been shown promising potential. The principal mechanism of therapeutic action has been partially ascribed to their strong paracrine capacity. Exosomes are small vesicles released from all kinds of cells and mediate intercellular communication by transferring exosomal protein and microRNA (miRNA) cargoes between cells in the brain. Among these cargoes, miRNAs play a key role in mediating biological function due to their prominent roles in gene regulation. Emerging data suggest that stem cell-released exosomes have advantages over stem cells to treat stroke, because exosomes could cross the blood bran barrier and easily to be modified and handled. Here, we first review the biogenesis, cargoes, and detection of exosomes. Then, we discussed the role of miRNAs in stroke. At last, we highlight the use of stem cell-released exosomes as biomarkers and therapeutic avenues in stroke. Perspectives on the developing role of stem cell-released exosomes mediated transfer of miRNAs as a therapeutic approach will also be discussed.

3.2931 **Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246**
Cooks, T., Pateras, J.S., Jenkins, L.M., Patel, K.M., Robles, A.I., Morris, J., Forshew, T., Appella, E., Gorgoulis, V.G. and Harris, C.C.
*Nature Communications, **9**:77 (2018)*

TP53 mutants (mutp53) are involved in the pathogenesis of most human cancers. Specific mutp53 proteins gain oncogenic functions (GOFs) distinct from the tumor suppressor activity of the wild-type protein. Tumor-associated macrophages (TAMs), a hallmark of solid tumors, are typically correlated with poor prognosis. Here, we report a non-cell-autonomous mechanism, whereby human mutp53 cancer cells reprogram macrophages to a tumor supportive and anti-inflammatory state. The colon cancer cells harboring GOF mutp53 selectively shed miR-1246-enriched exosomes. Uptake of these exosomes by neighboring macrophages triggers their miR-1246-dependent reprogramming into a cancer-promoting state. Mutp53-reprogammed TAMs favor anti-inflammatory immunosuppression with increased activity of TGF-β. These findings, associated with poor survival in colon cancer patients, strongly support a microenvironmental GOF role for mutp53 in actively engaging the immune system to promote cancer progression and metastasis.

3.2932 **Ursodeoxycholyl lysophosphatidylethanolamide negatively regulates TLRmediated lipopolysaccharide response in human THP-1-derived macrophages**
Horvatova, A., Utaipan, T., Otto, A-C., Zhang, Y., Gan-Schreier, H., Pavek, P., Pathil, A., Stremmel, W. and Chamulitrat, W.
*Eur. J. Pharmacol., **825**, 63-74 (2018)*

The bile acid-phospholipid conjugate ursodeoxycholyl oleoyl-lysophophatidylethanolamide (UDCA-18:1LPE) is an anti-inflammatory and anti-fibrotic agent as previously shown in cultured hepatocytes and hepatic stellate cells as well as in in vivo models of liver injury. We hypothesize that UDCA-18:1LPE may directly inhibit the activation of immune cells. We found that UDCA-18:1LPE was capable of inhibiting the migration of phorbol ester-differentiated human THP-1 cells. We examined anti-inflammatory activity of UDCA-18:1LPE during activation of THP1-derived macrophages. Treatment of these macrophages by bacterial lipopolysaccharide (LPS) for 24 h induced the release of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β. This release was markedly inhibited by pretreatment with UDCA-18:1LPE by ~ 65–90%. Derivatives with a different fatty-acid chain in LPE moiety also exhibited anti-inflammatory property. Western blotting and indirect immunofluorescence analyses revealed that UDCA-18:1LPE attenuated the expression of phosphorylated p38, MKK4/MKK7, JNK1/2, and c-Jun as well as nuclear translocation of NF-κB by ~ 22–86%. After LPS stimulation, the Toll-like receptor adaptor proteins, myeloid differentiation factor 88 and TNF receptor associated factor 6, were recruited into lipid rafts and UDCA-18:1LPE inhibited this recruitment by 22% and 58%, respectively. Moreover, LPS treatment caused a decrease of the known cytoprotective lysophosphatidylcholine species containing polyunsaturated fatty acids by 43%, and UDCA-18:1LPE co-treatment reversed this decrease. In conclusion, UDCA-18:1LPE and derivatives inhibited LPS inflammatory response by interfering with Toll-like receptor signaling in lipid rafts leading to an inhibition of MAPK and NF-κB activation. These conjugates may represent a class of lead compounds for development of anti-inflammatory drugs.

3.2933 **Chemotherapy induces secretion of exosomes loaded with heparanase that degrades extracellular matrix and impacts tumor and host cell behavior**
Bandari, S.K., Purushothaman, A., Ramani, V.C., Brinkley, G.J., Chandrashekar, D.S., varambally, S., Mobley, J.A., Zhang, Y., Brown, E.E., Vlodavsky, I. and Sanderson, R.D.
Matrix Biol., ***65***, *104-118 (2018)*

The heparan sulfate-degrading enzyme heparanase promotes the progression of many cancers by driving tumor cell proliferation, metastasis and angiogenesis. Heparanase accomplishes this via multiple mechanisms including its recently described effect on enhancing biogenesis of tumor exosomes. Because we recently discovered that heparanase expression is upregulated in myeloma cells that survive chemotherapy, we were prompted to investigate the impact of anti-myeloma drugs on exosome biogenesis. When myeloma cells were exposed to the commonly utilized anti-myeloma drugs bortezomib, carfilzomib or melphalan, exosome secretion by the cells was dramatically enhanced. These chemotherapy-induced exosomes (chemoexosomes) have a proteome profile distinct from cells not exposed to drug including a dramatic elevation in the level of heparanase present as exosome cargo. The chemoexosome heparanase was not found inside the chemoexosome, but was present on the exosome surface where it was capable of degrading heparan sulfate embedded within an extracellular matrix. When exposed to myeloma cells, chemoexosomes transferred their heparanase cargo to those cells, enhancing their heparan sulfate degrading activity and leading to activation of ERK signaling and an increase in shedding of the syndecan-1 proteoglycan. Exposure of chemoexosomes to macrophages enhanced their secretion of TNF-α, an important myeloma growth factor. Moreover, chemoexosomes stimulated macrophage migration and this effect was blocked by H1023, a monoclonal antibody that inhibits heparanase enzymatic activity. These data suggest that anti-myeloma therapy ignites a burst of exosomes having a high level of heparanase that remodels extracellular matrix and alters tumor and host cell behaviors that likely contribute to chemoresistance and eventual patient relapse.

3.2934 **Manufacturing Exosomes: A Promising Therapeutic Platform**
Colao, I.L., Corteling, R., Bracewell, D. and Wall, I.
Trend in Mol. Med., ***24(3)***, *242-256 (2018)*

Exosome research has been rejuvenated in recent years, due in part to the evolution in understanding of stem cell mode of action. The paracrine effect of stem cell therapy candidates has been mechanistically linked to inherited, specific functionality in secreted exosome derivatives.
Even though exosomes are expected to enter clinical trials imminently, there has been a lack of manufacturing process development work that is needed to generate clinically relevant quantities of exosomes as trials progress towards larger patient numbers.
If manufacturing research is not undertaken now, then the advancement of exosomes as a new therapeutic platform will be slowed. Thus, there is an urgent need for technological advancements.
Here, we present process options for industrial and academic researchers to consider to translate exosomes into viable therapeutic candidates from a manufacturing perspective.

3.2935 **Proteasome inhibition blocks necroptosis by attenuating death complex aggregation**
Ali, M. and Mocarski, E.S.
Cell Death & Disease, ***9***:*346 (2018)*

Proteasome inhibitors have achieved clinical success because they trigger intrinsic and extrinsic cell death to eliminate susceptible human cancers. The ubiquitin-proteasome protein degradation system regulates signaling pathways by controlling levels of components such as cellular inhibitor of apoptosis (cIAP)1 and cIAP2 in TNF-mediated cell death. Here, we sought to evaluate the contribution of necroptosis to the cell death pattern induced by the specific proteasome inhibitor Carfilzomib (Cf). Proteasome inhibitor-sensitive multiple myeloma cell lines die in response to Cf by apoptosis in combination with serine protease-dependent death, without any contribution of RIPK3-dependent necroptosis. Proteasome inhibition leads to the induction of apoptotic markers such as activated caspase-3 rather than necroptotic markers such as phosphorylated-MLKL in all cell lines tested. In HT-29 cells, Cf attenuates the late RIPK1 interaction with TNFR1 during TNF-induced necroptosis without altering the sensitivity of cIAP antagonists. Cf treatment results in decreased translocation of death signaling components RIPK1, FADD, caspase-8, cFLIP, and RIPK3 to detergent insoluble fractions. Our results show that proteasome inhibition with Cf impairs necroptosis and favors apoptosis even in cells with intact necroptotic machinery. Following the induction of TNFR1-mediated necroptosis, proteasome activity stabilizes effective aggregation and activation of ripoptosome/necrosome complexes.

3.2936 Extracellular vesicles as a platform for membrane-associated therapeutic protein delivery
Yang, Y., Hong, Y., Cho, E., Kim, G.B. and Kim, I-S.
*J. Extracellular Vesicles, **7**:1, 1440131 (2018)*

Membrane proteins are of great research interest, particularly because they are rich in targets for therapeutic application. The suitability of various membrane proteins as targets for therapeutic formulations, such as drugs or antibodies, has been studied in preclinical and clinical studies. For therapeutic application, however, a protein must be expressed and purified in as close to its native conformation as possible. This has proven difficult for membrane proteins, as their native conformation requires the association with an appropriate cellular membrane. One solution to this problem is to use extracellular vesicles as a display platform. Exosomes and microvesicles are membranous extracellular vesicles that are released from most cells. Their membranes may provide a favourable microenvironment for membrane proteins to take on their proper conformation, activity, and membrane distribution; moreover, membrane proteins can cluster into microdomains on the surface of extracellular vesicles following their biogenesis. In this review, we survey the state-of-the-art of extracellular vesicle (exosome and small-sized microvesicle)-based therapeutics, evaluate the current biological understanding of these formulations, and forecast the technical advances that will be needed to continue driving the development of membrane protein therapeutics.

3.2937 The dynamic recruitment of TRBP to neuronal membranes mediates dendritogenesis during development
Antoniou, A., Khudayberdiev, S., Idziak, A., Bicker, S., Jacob, R. and Schratt, G.
*EMBO Reports, **19**, e44853 (2018)*

MicroRNAs are important regulators of local protein synthesis during neuronal development. We investigated the dynamic regulation of microRNA production and found that the majority of the microRNA-generating complex, consisting of Dicer, TRBP, and PACT, specifically associates with intracellular membranes in developing neurons. Stimulation with brain-derived neurotrophic factor (BDNF), which promotes dendritogenesis, caused the redistribution of TRBP from the endoplasmic reticulum into the cytoplasm, and its dissociation from Dicer, in a Ca^{2+}-dependent manner. As a result, the processing of a subset of neuronal precursor microRNAs, among them the dendritically localized pre-miR16, was impaired. Decreased production of miR-16-5p, which targeted the BDNF mRNA itself, was rescued by expression of a membrane-targeted TRBP. Moreover, miR-16-5p or membrane-targeted TRBP expression blocked BDNF-induced dendritogenesis, demonstrating the importance of neuronal TRBP dynamics for activity-dependent neuronal development. We propose that neurons employ specialized mechanisms to modulate local gene expression in dendrites, via the dynamic regulation of microRNA biogenesis factors at intracellular membranes of the endoplasmic reticulum, which in turn is crucial for neuronal dendrite complexity and therefore neuronal circuit formation and function.

3.2938 Phagocytosis depends on TRPV2-mediated calcium influx and requires TRPV2 in lipids rafts: alteration in macrophages from patients with cystic fibrosis
Leveque, M., penna, A., Le Trionnaire, S., Belleguic, C., Desrues, B., Brinchault, G., Jouneau, S., Lagadic-Gossmann, D. and Martin-Chouly, C.
*Scientific Reports, **8**:4310 (2018)*

Whereas many phagocytosis steps involve ionic fluxes, the underlying ion channels remain poorly defined. As reported in mice, the calcium conducting TRPV2 channel impacts the phagocytic process. Macrophage phagocytosis is critical for defense against pathogens. In cystic fibrosis (CF), macrophages have lost their capacity to act as suppressor cells and thus play a significant role in the initiating stages leading to chronic inflammation/infection. In a previous study, we demonstrated that impaired function of CF macrophages is due to a deficient phagocytosis. The aim of the present study was to investigate TRPV2 role in the phagocytosis capacity of healthy primary human macrophage by studying its activity, its membrane localization and its recruitment in lipid rafts. In primary human macrophages, we showed that P. aeruginosa recruits TRPV2 channels at the cell surface and induced a calcium influx required for bacterial phagocytosis. We presently demonstrate that to be functional and play a role in phagocytosis, TRPV2 might require a preferential localization in lipid rafts. Furthermore, CF macrophage displays a perturbed calcium homeostasis due to a defect in TRPV2. In this context, deregulated TRPV2-signaling in CF macrophages could explain their defective phagocytosis capacity that contribute to the maintenance of chronic infection.