APPLICATION NOTE

Towards Understanding the Molecular Basis of Parkinson's Disease: Cell-based Model of Mitophagy and Aggresome Accumulation

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CYTO-ID® AUTOPHAGY DETECTION KIT (ENZ-51031)
MITO-ID® RED DETECTION KIT (ENZ-51007)
PROTEOSTAT® AGGRESOME DETECTION KIT (ENZ-51035)

INTRODUCTION

Parkinson's disease (PD) is an incapacitating, age-related neurodegenerative disease, affecting all populations, with approximately 1.5 million confirmed cases in the United States alone. Data from various studies of genes associated with hereditary disease, toxicology studies using animal models/*in vitro* models and also patient-based studies have implicated compromised protein turnover relating to the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP), as well as diminished mitochondrial activity in the most common idiopathic forms of the disease. Mutations in the Parkin gene are a primary cause of autosomal recessive juvenile PD. Parkin functions as an E3 ligase that ubiquitinylates α-synuclein, the primary aggregated protein associated with the neurotoxic accumulation in Lewy bodies. Parkin also interacts with and ubiquitinylates depolarized mitochondria, promoting their clearance by mitophagy. Cell-based assays relevant to monitoring aspects of PD are presented, including aberrant aggresome formation, mitochondrial depolarization, ubiquitinylation and mitophagy. The described assays should contribute to the understanding of regulatory pathways controlling mitophagy and Lewy body formation, aiding in the characterization of various human neuropathological disorders, including PD.

BACKGROUND

After Alzheimer's disease, Parkinson's disease (PD) is the 2nd most common neurodegenerative disorder (> 6 million cases worldwide). Many genes mutated in hereditary PD play a pathogenic role in mitochondria as well as in the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP), both of which dispose of misfolded proteins and damaged organelles. α-synuclein association with the inner mitochondrial membrane coincides with selective age-related mitochondrial complex I inhibition and decreased respiration, along with increased mitophagy. α-synuclein is the major misfolded protein of Lewy bodies, a fundamental pathological feature of the degenerating PD brain.

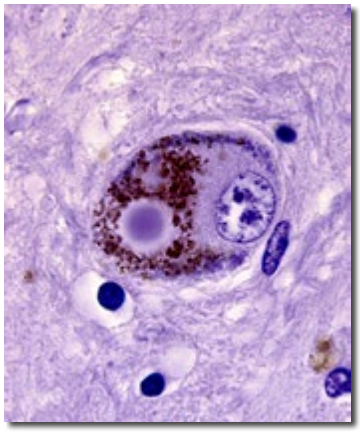


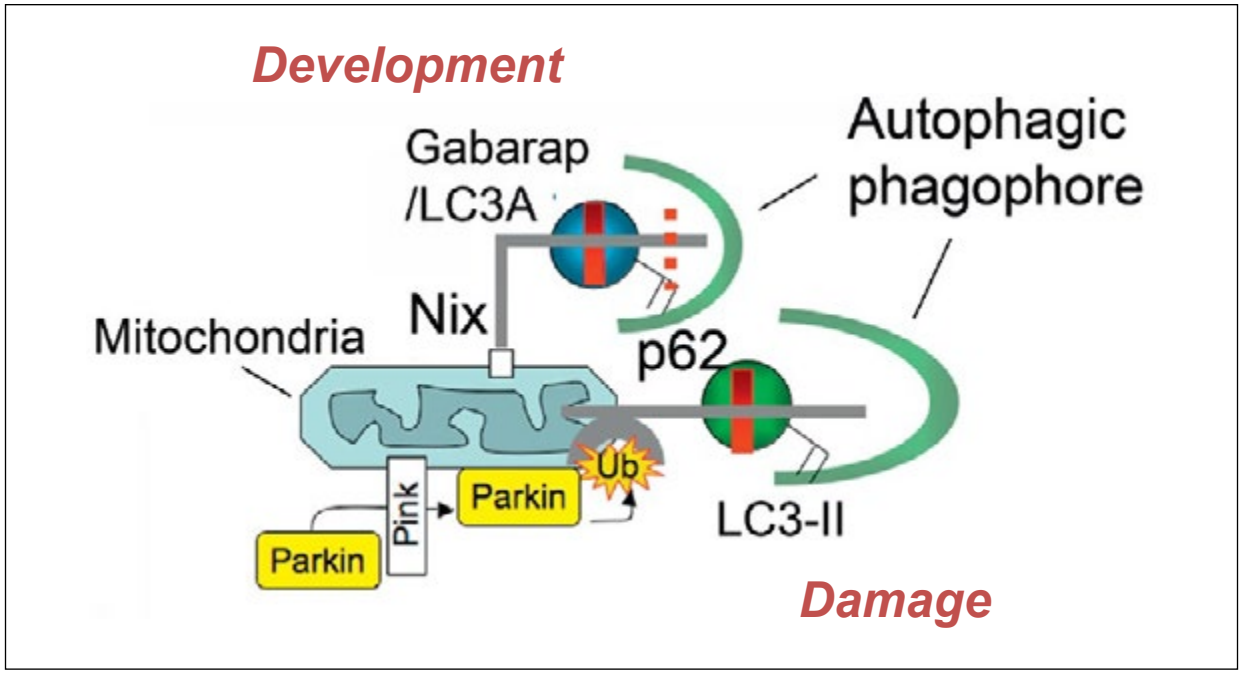
Figure 1: Lewy body in a *substantia nigra* neuron

ROLE OF UBIQUITIN IN PARKINSON'S DISEASE

Parkin is a RING-type E3 ubiquitin-protein ligase that triggers selective ubiquitinylation and targeting of depolarized mitochondria for sequestration in aggresomes and/or autophagosomes, leading to degradation by the ALP. Parkin ubiquitinylates Hsp70 on multiple residues, unrelated to Hsp70 degradation. Lewy bodies are positive for molecular chaperones suggesting they play a role in progression of PD. Interference with chaperone activity accelerates α-synuclein toxicity.

Another parkin substrate, Synphilin-1, interacts with α-synuclein promoting its aggregation. Synphilin-1 prevents α-synuclein-degradation by the UPS and this is reversed by targeting of synphilin-1 to degradation through ubiquitinylation. α-synuclein itself interacts and co-localizes with Siah-1, another RING-type E3 ubiquitin-protein ligase. α-synuclein- and ubiquitin-positive inclusion bodies are the pathological hallmarks of PD.

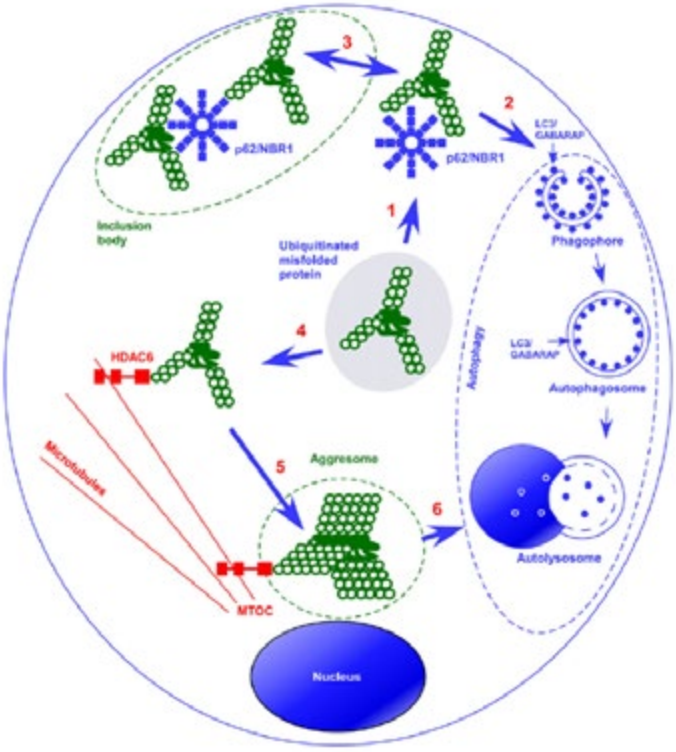
MOLECULAR ADAPTERS TARGETING MITOCHONDRIA FOR AUTOPHAGIC DEGRADATION



Ref. Deretic V. F1000 Biol Rep. (2010) Jun 23;2. pii: 45.

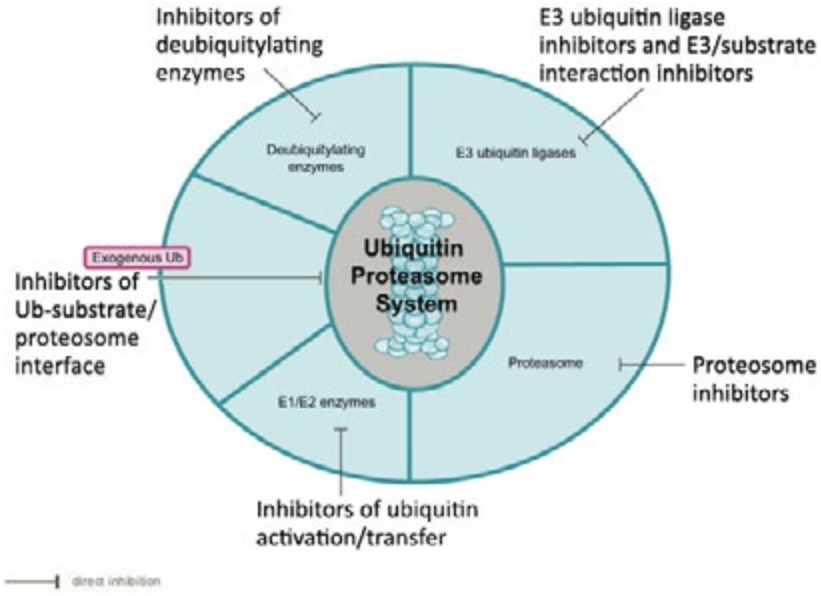
DEGRADATION OF PROTEIN AGGREGATES BY AUTOPHAGY

1. Aggregated proteins are ubiquitinylated and recognized by the oligomeric p62 and NBR1 proteins.
2. This targets the proteins for selective degradation by autophagy
3. p62 and NBR1 also mediate formation of proteinacious inclusion bodies.
4. Binding of HDAC6 to ubiquitinylated proteins ensures their transport along the microtubules toward the MTOC.
5. Excess misfolded proteins can then be organized into aggresomes.
6. Inclusion bodies and aggresomes may serve as depots for later autophagic degradation of stored misfolded proteins.



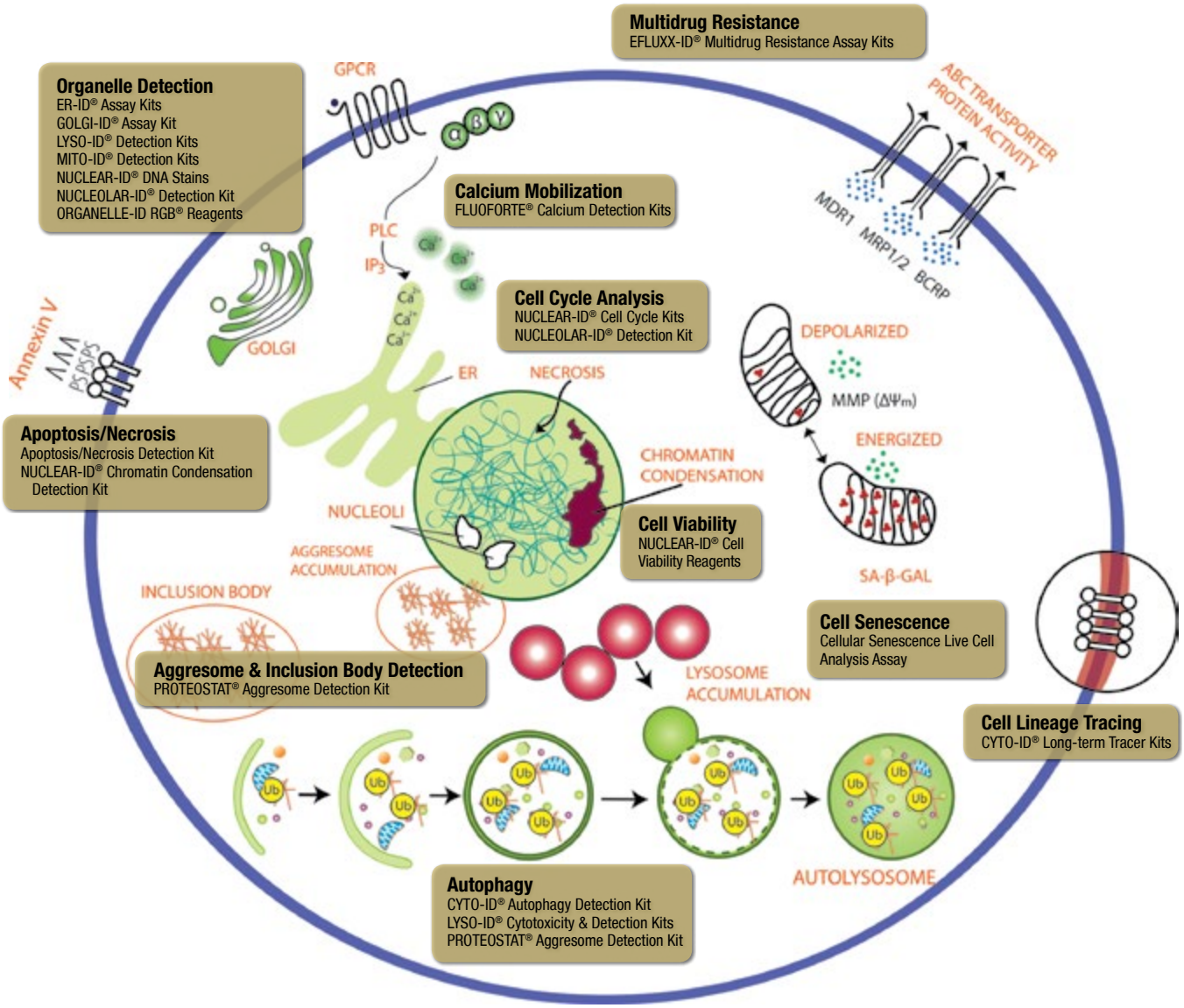
[Closed boxes, Ub-binding domains; empty circles, Ub; filled circles, conjugated LC3/GABARAP proteins.]
Ref. Kirkin et al Mol Cell. (2009) 34(3):259-69.

POTENTIAL DRUG TARGETS FOR PARKINSON'S DISEASE RELATED TO UBIQUITIN



Ref. Guédat P, Colland F. BMC Biochem. (2007) Nov 22;8 Suppl 1:S14.

PHENOTYPIC ANALYSIS WITH ORGANELLE & PATHWAY-TARGETING CELLESTIAL® DYES

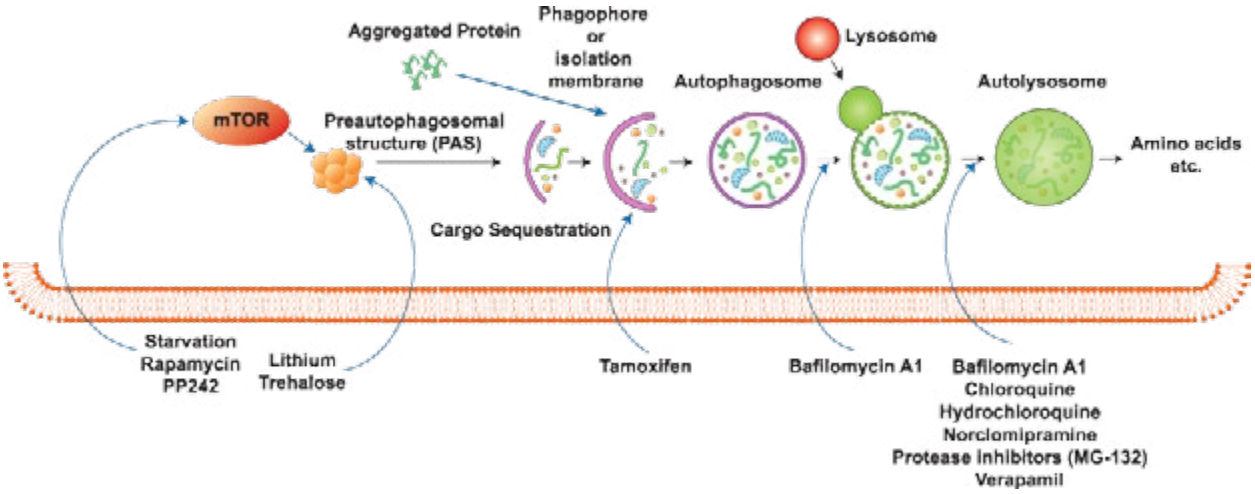


PHENOTYPIC ASSAYS RELEVANT TO SCREENING OF SMALL MOLECULES IN PD DRUG DISCOVERY

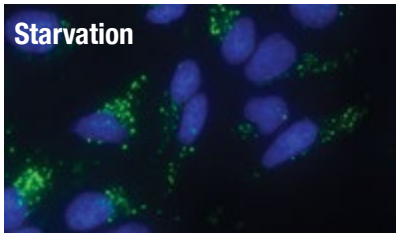
Objective: Develop a compendium of phenotypic assays suitable for monitoring aspects of PD *in vitro* and *in situ*.

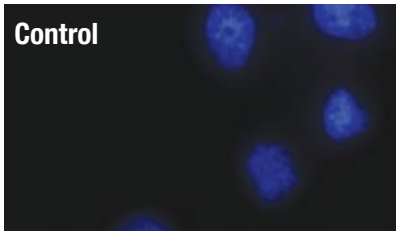
- Autophagy: CYTO-ID® Green Autophagy Detection Kit.
- Mitochondrial Degradation: MITO-ID® Red Detection Kit
- Aggresomes, Inclusion Bodies and Lewy Bodies: PROTEOSTAT® Aggresome Detection Kit

CYTO-ID® AUTOPHAGY DYE FOR LIVE CELL ANALYSIS BY MICROSCOPY AND FLOW CYTOMETRY



Treatment	Mean (FL1) Signal
Control	40.7
1 hour Starvation and 1 hour Recovery	41.6
1 hour Starvation	104





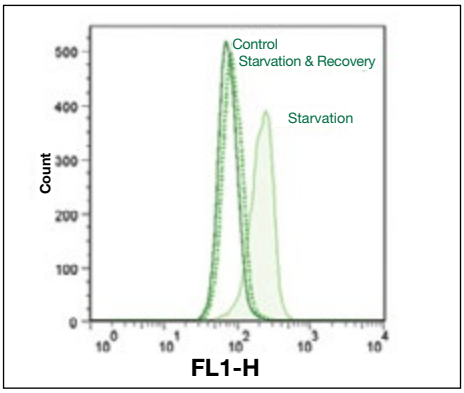


Figure 2: CYTO-ID® green signal increases about 2.5-fold after 1 hr starvation. Re-feeding reverses signal increase.

MITO-ID® RED DYE FOR LIVE OR FIXED CELL ANALYSIS BY MICROSCOPY

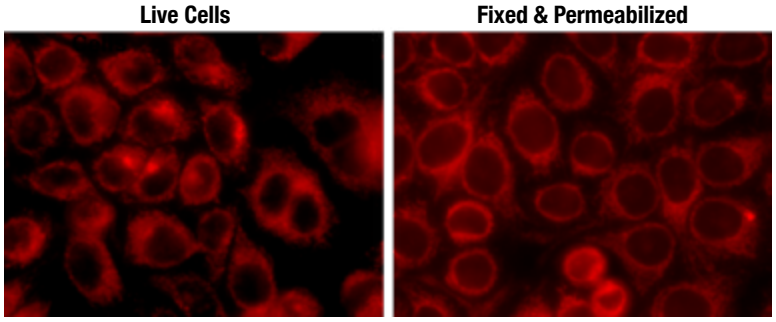


Figure 3: Live cell staining allows multiplexing with green probes (CYTO-ID® Autophagy dye, GFP). Fixed cell staining allows multiplexing with fluorescent antibody conjugates. MitoTracker® Red dye performs poorly in fixed cells.

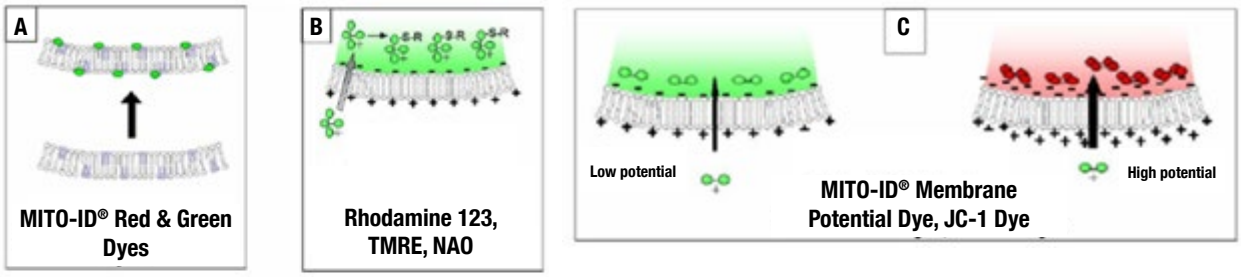
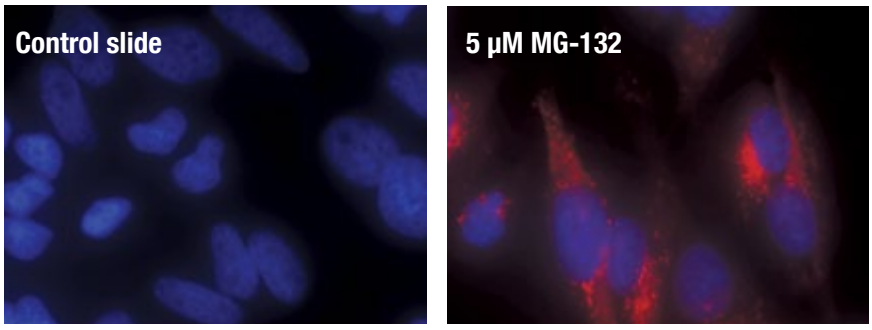
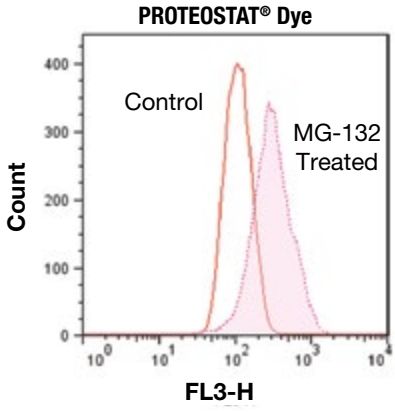


Figure 4: Cell-Permeable Mitochondria-Selective Dyes:
A. Those that bind to structures in the inner mitochondrial membrane.
B. Those that concentrate inside mitochondria, as membrane potential increases.
C. Those that reversibly change color as membrane potential increases (dual-emission potential probes).

PROTEOSTAT® AGGRESOME DYE FOR FIXED CELL ANALYSIS BY MICROSCOPY AND FLOW CYTOMETRY

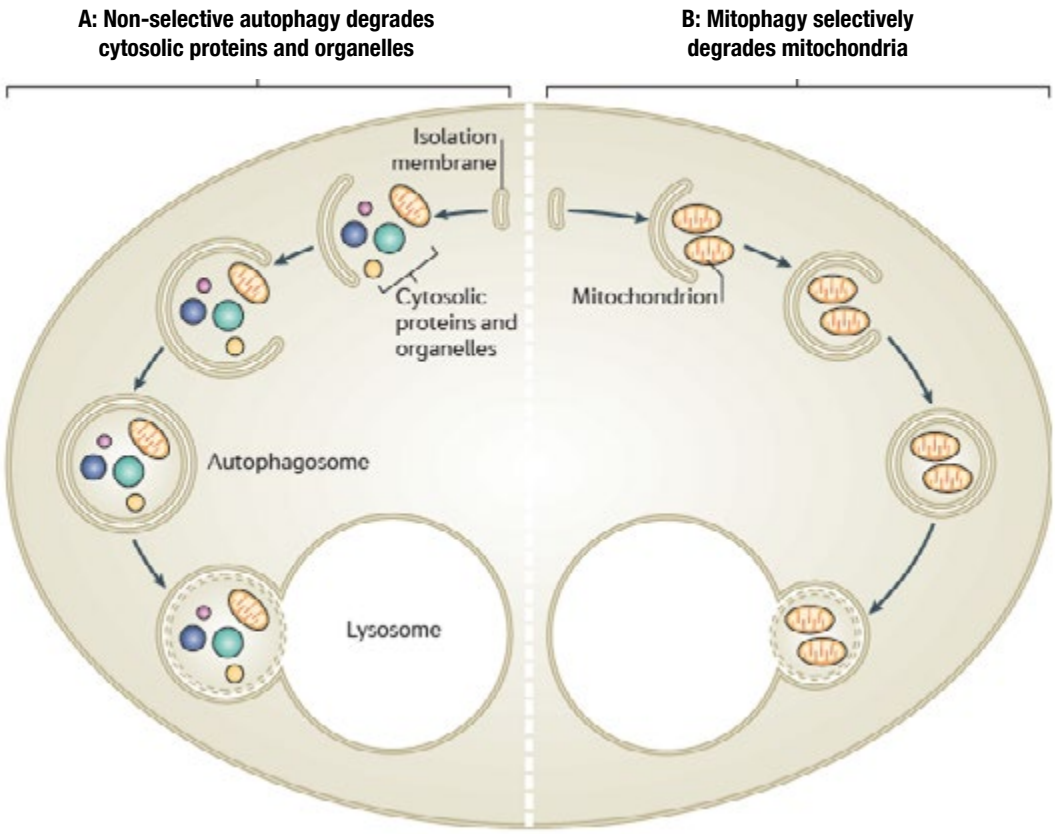




- Misfolded and aggregated proteins are ubiquitinylated and stored in aggresomes as well as other inclusion bodies.
- Subsequently, they may be shuttled into the autophagy pathway for degradation.
- Phenomenon is relevant to cancer therapeutics (Velcade®) and neurodegenerative disease.

Treatment	Mean (FL3) Signal
Control	113
Treated (5 µM MG132)	335
Aggresome Propensity Factor	66

BENCHMARKING MITOCHONDRIAL DEGRADATION PATHWAYS



Ref: Youle & Narendra, NATURE REVIEWS | Molecular Cell Biology (2011), 12, 9-14

SERUM STARVATION STIMULATES MACROAUTOPHAGY

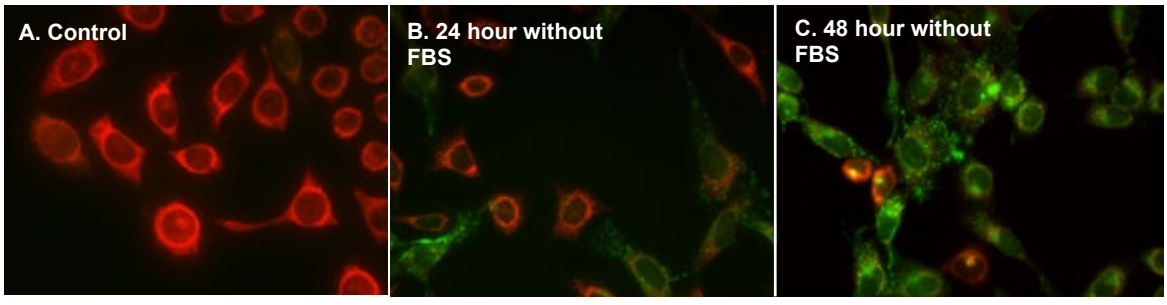


Figure 5: Control HeLa cells were incubated in Earl's Minimum Essential medium (EMEM) with 10% fetal bovine serum (A.) and starved cells were generated by incubation in Earl's Minimum Essential medium (EMEM) with 0% fetal bovine serum (B. and C.) at 37°C for the indicated time periods. Following this incubation period, both control and starved cells were incubated with CYTO-ID® Green and MITO-ID® Red dyes for 15 minutes at 37°C and then washed with assay buffer.

CELL-BASED MODEL FOR MITOPHAGY

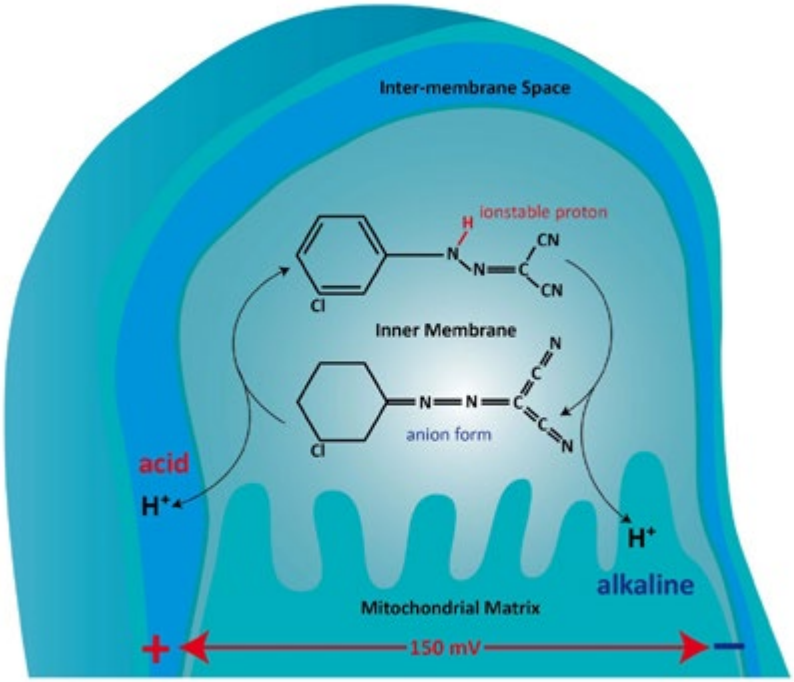


Figure 6: Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), is a chemical inhibitor of oxidative phosphorylation. CCCP causes an uncoupling of the proton gradient that is established during the normal activity of electron carriers in the electron transport chain. The compound acts essentially as an ionophore and reduces the ability of ATP synthase to function optimally. Parkin-transfected HeLa cells were treated with 10 µM CCCP overnight to induce mitochondrial dysfunction.

PARKIN REDISTRIBUTES UPON LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL

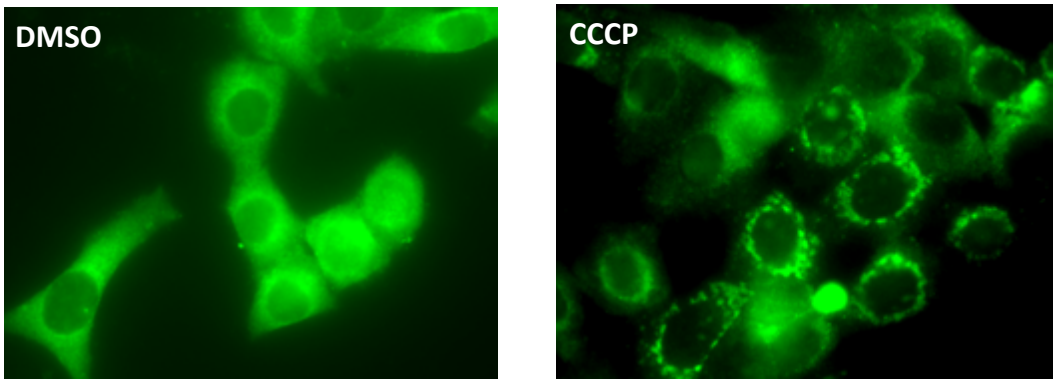


Figure 7: HeLa cells were transfected with Parkin (PARK2) for 1 hour and then treated with DMSO (control) or CCCP (10 µM) for 1 h. Both control and CCCP treated cells were fixed, permeabilized, and immunostained with Alexa Fluor 488 dye-labeled Parkin Ab. Under basal conditions, parkin is diffusely distributed in the cytosol. Following loss of mitochondrial membrane potential, a dramatic redistribution of parkin is observed.

PARKIN ACCUMULATES & CO-LOCALIZES WITH DE-ENERGIZED MITOCHONDRIA

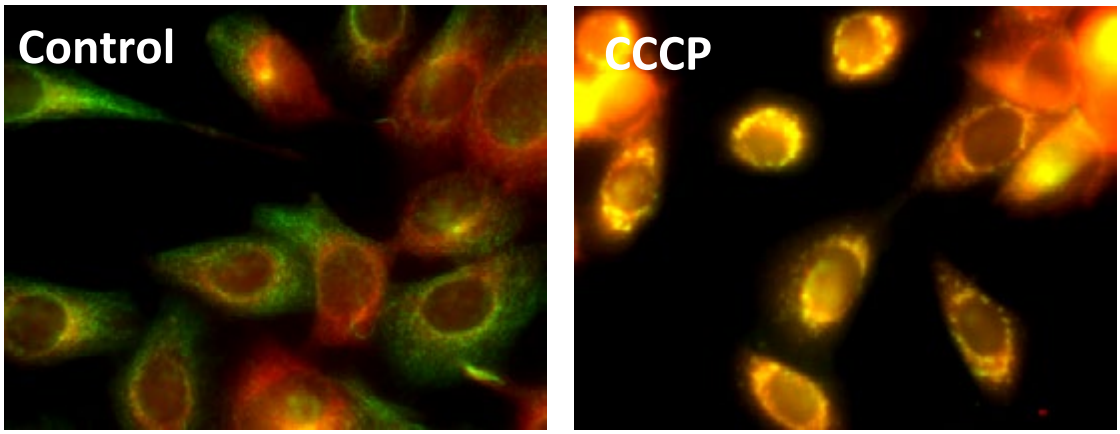


Figure 8: HeLa cells were transfected with Parkin for 1 hour and then treated with DMSO (control) or CCCP (10 uM) for 1 hour. Both control and CCCP treated cells were fixed and permeabilized, and stained with Alexa Fluor 488 dye-labeled parkin Ab, followed by MITO-ID® Red dye. Co-localization of parkin and mitochondria is evident.

PARKIN UBIQUITINYLATES MITOCHONDRIA UPON LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL

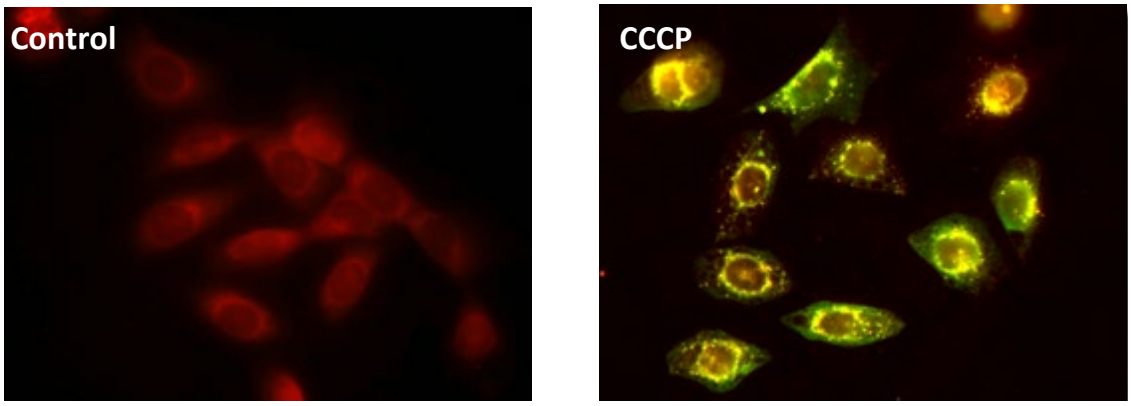


Figure 9: HeLa cells were transfected with Parkin for 1 hour and then treated with DMSO (control) or CCCP (10 µM) for 24 hour. The cells were fixed and immunostained using Atto 488 dye-labeled ubiquitin antibody and MITO-ID® Red dye. Parkin promotes mitochondrial ubiquitinylation following CCCP treatment.

MITOCHONDRIA ARE ELIMINATED BY MITOPHAGY UPON DEPOLARIZATION & UBIQUITINYLATION

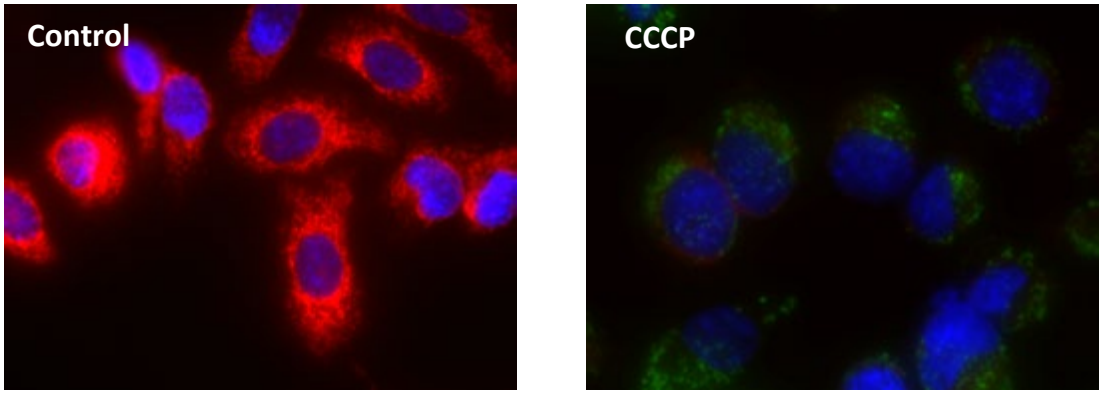


Figure 10: HeLa cells transfected with Parkin were treated with DMSO (control) or 10 µM CCCP for 24 h, respectively. Following this incubation period, both control and treated cells were incubated with CYTO-ID® Green, MITO-ID® Red and Hoechst dye for 15 minutes at 37°C and then washed with assay buffer. Parkin induces elimination of de-energized mitochondria by mitophagy.

AGGRESOMES ACCUMULATE WITHIN CELLS UNDERGOING MITOPHAGY

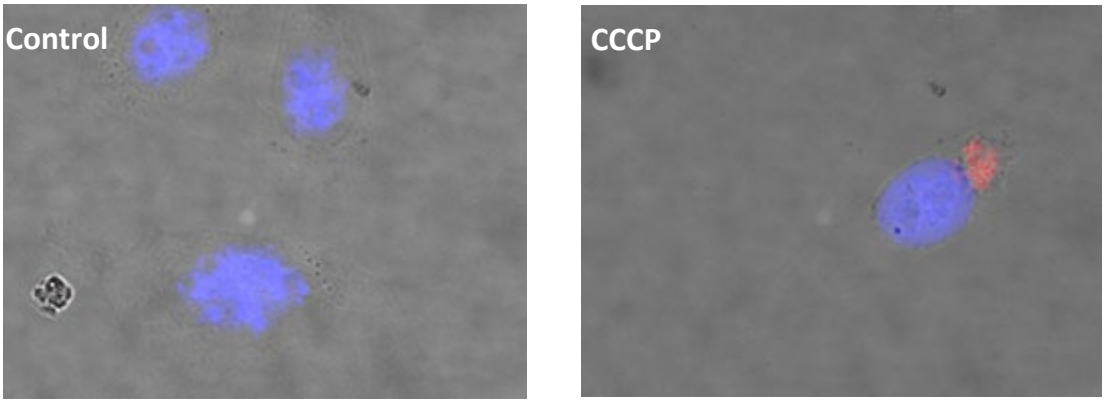


Figure 11: Composite bright-field and fluorescence microscopy images: HeLa cells were transfected with Parkin for 24 hour and then treated with DMSO (control) or CCCP (10 µM) overnight. Both control and CCCP-treated cells were fixed and permeabilized, then stained with Hoechst dye and PROTESTAT® Aggresome Detection Dye. Aggregated proteins coalesce in peri-nuclear Lewy body-like structures within cells undergoing mitophagy.

CONCLUSIONS

- The pathophysiology of PD is intricately entwined with ubiquitinylation, through the UPS and ALP.
- Various CELLESTIAL® dyes can readily be implemented in phenotypic assays targeting different aspects of PD.
- The phenotypic assays could potentially be used to identify NCEs that selectively target PD-associated E3 ubiquitin-protein ligases, such as parkin.

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