Determining how disease-associated mutations affect the dynamics of mitochondrial recruitment and loss of the mitophagy regulator, Parkin

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Introduction

Mitophagy is a mitochondrial quality control process that regulates the destruction of damaged, depolarized mitochondria and is associated with neurodegenerative disorders such as Parkinson's Disease (PD). Parkin and PTEN-induced kinase 1 (PINK1) are two proteins that are crucial to mitophagy (Deas *et al*., 2011).

At depolarized mitochondria, PINK1 accumulates on the outer mitochondrial membrane (OMM), recruiting and activating the cytosolic E3 ubiquitin ligase, Parkin, which conjugates polyubiquitin (pUb) on OMM substrates. These pUb chains are phosphorylated by PINK1, creating phospho-polyubiquitin (ppUb), a unique marker of damaged mitochondria (Kondapalli *et al*., 2012; Sarraf *et al*., 2013). The ppUb serve as a platform to recruit additional Parkin and autophagy receptors that facilitate the recruitment of the autophagic machinery and lead to the isolation of mitochondria within autophagosomes (Figure 1; Shiba-Fukushima *et al*., 2014). However, if mitochondria are repolarized at a sufficiently early stage, PINK1 will rapidly dissociate, followed later by Parkin, effectively aborting the process (Bowling *et al*., 2019).

Disease-associated mutations in Parkin have been shown to affect both its E3 ubiquitin ligase activity and recruitment to depolarized mitochondria, but it is currently unclear whether these also affect the release of Parkin from the OMM after repolarization. In this study, we investigate the effects of 3 different Parkin mutations on this process.

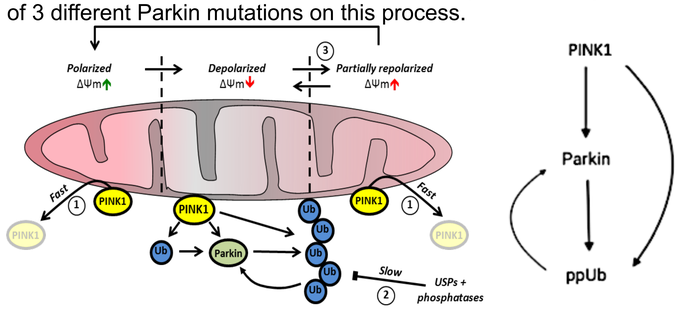


Figure 1. PINK1:Parkin Mitophagy Pathway. Diagrammatic representation of the PINK1:Parkin pathway (left) and the interlinked PINK1:Parkin coherent feedforward and postive feedback loops associated with ppUb production (Bowling et al.).

The mutations involved in our study are R275W, C431S, and W403A.

- **C431S**: Lacks E3 ligase activity as essential catalytic cysteine is mutated to serine. *Hypothesis:* it will slowly associate and then rapidly dissociate from the OMM upon repolarization.
- **R275W**: Partial loss of E3 ligase activity due to mutation in RING domain. Is recruited at the same rate as wildtype (WT) Parkin. *Hypothesis*: it will quickly dissociate from Parkin after repolarization
- **W403A**: hyperactive E3 ligase mutant that is recruited to mitochondria with identical kinetics to WT Parkin. *Hypothesis:* it will dissociate at a much slower rate.

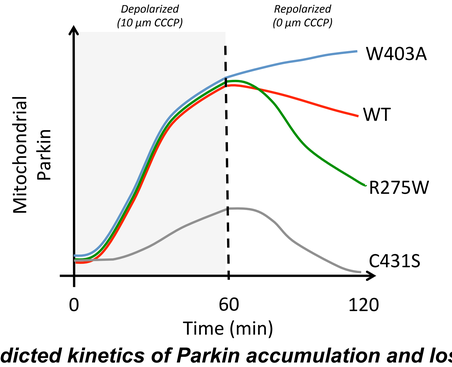


Figure 2. Predicted kinetics of Parkin accumulation and loss at the OMM after depolarization and subsequent repolarization of the mitochondria.

Mutant Generation and Verification

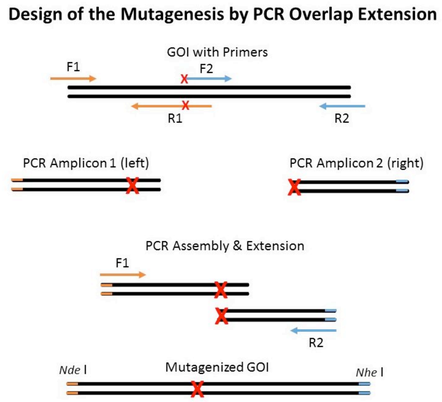


Figure 3. PCR Mutagenesis Design. A plasmid containing the human WT Parkin cDNA was used as a template and primers were designed to generate two overlapping products with the desired mutation in the overlapping region. These fragments were combined by PCR to produce products encoding full-length mutant Parkin. This was introduced into a plasmid in frame with EYFP (enhanced yellow fluorescent protein) at the N-terminus using conventional cloning techniques (Civicbio.com).

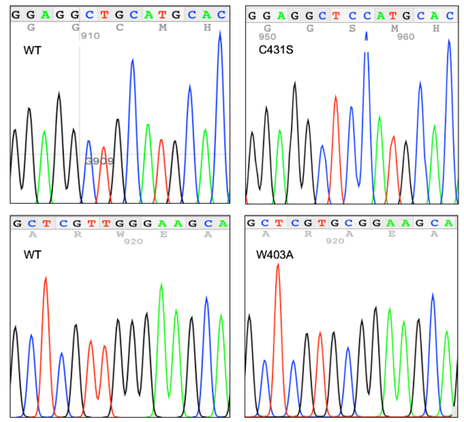
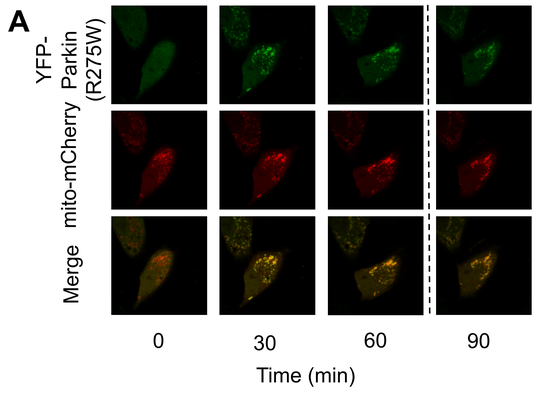


Figure 4. C431S and W403A Mutant Verification. Having generated pEYFP-Parkin C431S and W403A expression plasmids using the methods described in Fig. 3, the presence of the mutations was confirmed using Sanger Sequencing. Chromatographs for the C431S (top right) and W403A (bottom right) are shown together with the corresponding region of the WT Parkin cDNA sequence.

Live Cell Imaging Experiments



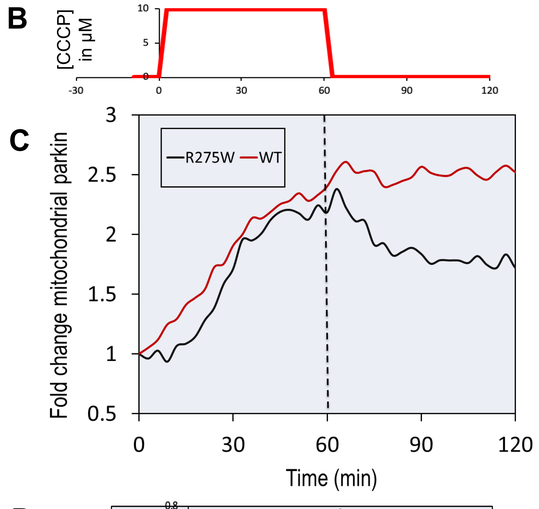


Figure 5. R275W & C431S dissociate from mitochondria after repolarization more rapidly than WT Parkin. HeLa cells were transiently transfected with plasmid constructs to express the fluorescent mitochondrial marker, mito-mCherry and EYFP fusions of WT or mutant Parkin proteins. The cells were depolarized with the reversible mitochondrial ionophore, CCCP for the first 60 minutes of the experiment and observed by live cell fluorescence microscopy. CCCP was washed out to repolarize the mitochondria and the cells were observed for a further 60 minutes. (A) Representative images from experiment using R275W YFP-Parkin (Jiwoo E. Park). (B) CCCP treatment regime used in experiments. (C) Quantification of the fold change in mitochondrial Parkin levels for the WT and R275W mutant. (Wesley A. Riley). (D) Quantification Parkin (WT or C431S) colocalization with mito-mCherry, as quantified by PCC analysis. In (C+D), dashed line represents time [CCCP] was decreased from 10 to 0 µM.

Discussion

- The PINK1:Parkin mitophagy pathway is a mitochondrial quality control mechanism in mammalian cells. Mutations in the gene encoding Parkin is associated with PD. **Figure 1** illustrates the relationship between PINK1, Parkin, and ppUb chains. Parkin is an E3 ubiquitin ligase that works in concert with PINK1 to create ppUb chains to mark depolarized mitochondria for degradation.
- The purpose of this project was to investigate the role of ppUb in the retention of Parkin proteins at mitochondria after the recovery of mitochondrial membrane potential. We hypothesized that PD-associated Parkin mutants that were E3-ligase dead would be released from mitochondria more rapidly that the WT protein (**Figure 2**).
- In order to generate mutant Parkin expression constructs, we used the 2-step PCR mutagenesis technique (**Figure 3)** and verified the mutations through DNA sequencing (**Figure 4**).
- We then expressed EYFP-tagged WT or mutant Parkin proteins together with a red fluorescent mitochondrial marker in HeLa cells. Live cell confocal microscopy was used to measure mitochondrial accumulation and loss of Parkin stimulated by a 60-minute pulse treatment with the reversible mitochondrial depolarizing agent, CCCP.
- We have gathered preliminary data comparing the dynamics of WT and R275W Parkin proteins by measuring fold change Parkin at the mitochondria (**Figure 5A-C**). In separate experiments, we used PCC colocalization analysis to compare the dynamics of WT and C431S Parkin proteins (**Figure 5D**). We intend to repeat these experiments and also perform similar experiments with the W403A hyperactive Parkin mutant.

References

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Acknowledgements

This work was supported by the MTSU MOBI, FRCAC, and URECA programs. Figures 1 and 2 were designed by DEN. The R275W mutant was constructed by WAR, the C431S and W403A mutants were constructed by JEP, and the mutant validation was completed by GSM and JEP. The R275W confocal microscopy images were recorded by JEP and the R275W fold change figure was supplied by WAR. Colocalization experiments and PCC analysis for the C431S mutant was supplied by GSM. Special thanks to J. Logan Bowling for technical assistance and support.