# Determining How the PINK1:Parkin Mitophagy Pathway Responds to Transient Mitochondrial Stress and How This is Affected By Disease Associated Mutations in 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 for mitophagy (Deas, Wood, & Plun-Favreau, 2011).

At depolarized mitochondria, PINK1 accumulates on the outer mitochondrial membrane (OMM), recruiting and activating the cytosolic E3 ubiquitin ligase, Parkin to conjugate polyubiquitin (pUb) on OMM substrates. These pUb are phosphorylated by PINK1, creating phospho-polyubiquitin (ppUb), a unique marker of damaged mitochondria (Kondapalli et al., 2012). These ppUb chains 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 terminating the process (Bowling et al., 2018).

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 the C431S Parkin mutation on this process.

**C431S**: Lacks E3 ligase activity as essential catalytic cysteine is mutated to serine. *Hypothesis:* C431S Parkin will slowly associate and then rapidly dissociate from the OMM upon repolarization.

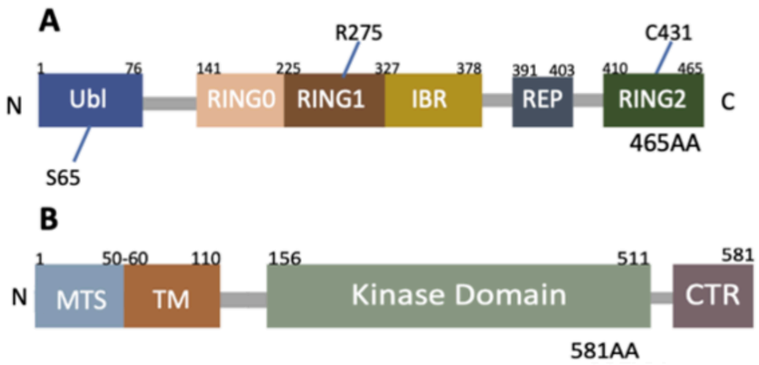


**Figure 1. Structure of PINK1 and Parkin protein.**
**A.** Parkin contains a Ubiquitin-like domain (Ubl), really interesting new gene domains (RING), in-between RINGs (IBR), and repressor element of Parkin domain (REP). Ser65 is the site of phosphorylation by PINK1. R275 is shown to be essential for E3 function. C431 is a catalytic cysteine which is essential for E3 ligase function. **B.** PTEN-induced putative kinase 1 (PINK1) contains a mitochondrial targeting sequence (MTS), transmembrane domain (TM), Kinase domain, and C-terminal repressor domain (CTR), which has an undefined start codon. (Adapted from Trempe and Fon, 2013).


**Figure 2. The PINK1: Parkin Mitophagy Pathway.**
A. Feed forward loop of PINK1: Parkin mitophagy pathway. B. Effect of mitochondrial membrane polarization on the mitophagy pathway. In healthy mitochondria, PINK1 is imported into mitochondria and degraded. When membrane potential is lost, PINK1 is stablized and accumulates on the OMM and recruits Parkin by phosphorylating pre-ubiquitinated substrate proteins. Parkin catalyzes the formation of ppUb chains which serves as anchors to the OMM. Following repolarization, PINK1 is degraded once again, ppUb chains are disassembled, and Parkin slowly delocalizes back to the cytosol (Adapted from Bowling et al 2019).

## Mutant Verification and Predicted Kinetics

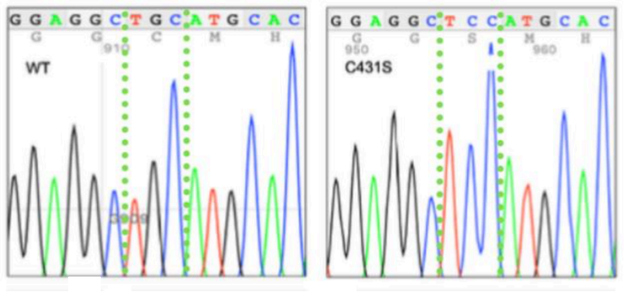


**Figure 3. C431S Mutant Verification.**
Having generated pEYFP-Parkin C431S expression plasmids using two-step PCR-based mutagenesis, the presence of the mutation was confirmed using Sanger Sequencing. The chromatograph is shown with the corresponding región of WT Parkin cDNA sequence.

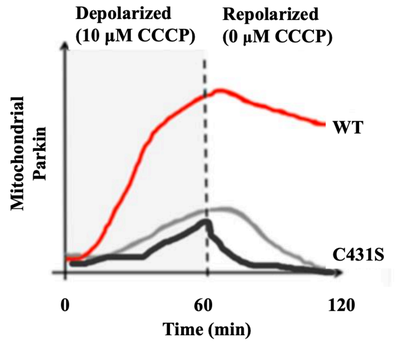


**Figure 4. Predicted kinetics of Parkin accumulation and loss after depolarization and subsequent repolarization of the mitocondria.**
If the hypothesis is correct, C431S Parkin will quickly dissociate from the OMM (black line), however, if our hypothesis is incorrect, we will see dissociation similar to WT Parkin (grey line).

## Live Cell Imaging Experiments

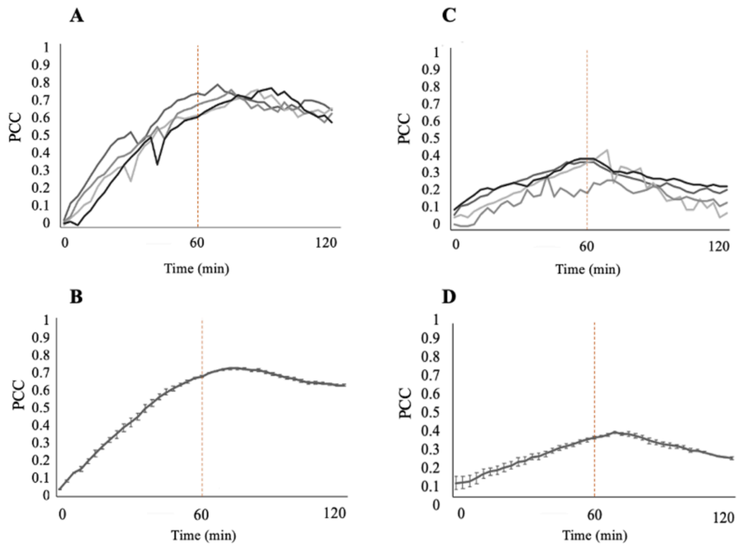


**Figure 5. PCC analysis of wildtype and C431S Parkin colocalization with the mitochondrial marker, mito-mCherry.**
Mitochondrial depolarization was induced utilizing the reversible depolarizing agent CCCP for 60 minutes before removal (dashed red line) and a repolarization phase (60-120 min). PCC Analysis was performed across 40 time points, every three minutes across two hours. **A.** Four representative cells expressing wildtype Parkin **B.** Wildtype Parkin average PCC values shown with standard errors. **C.** Four representative cells expressing C431S Parkin. **D.** C431S Parkin average PCC values shown with standard errors.

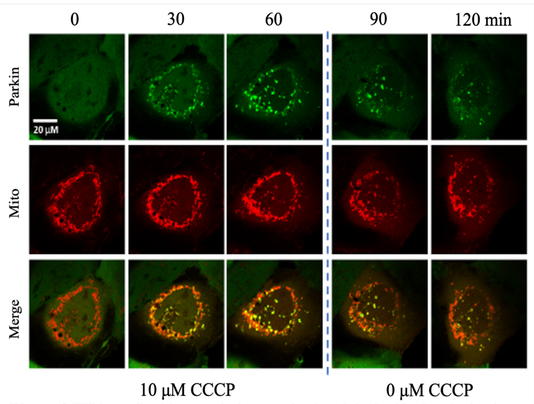


**Figure 6. Wildtype Parkin association to mitochondria during depolarization and repolarization of the mitochondrial membrane.**
HeLa cells expressing EYFP-Parkin and mito-mCherry were treated with 10 µM CCCP for one hour followed by removal at T=60 min. Time lapse images display Parkin recruitment to mitochondrial membrane during depolarization (10 µM CCCP) followed by retention during repolarization (0 µM CCCP). EYFP-Parkin (green) is seen associating at the mitocondria, with Mito-mcherry flourescent tagging (red).

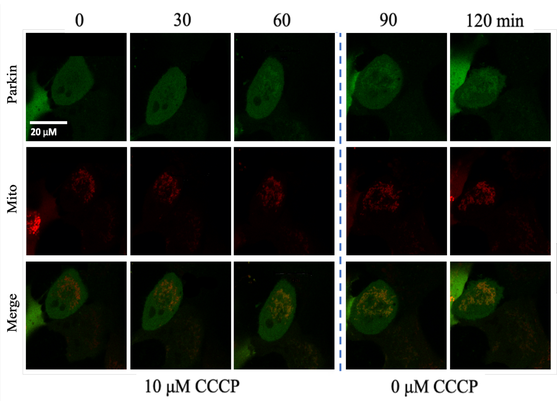


**Figure 7. Mutant Parkin retention following CCCP treatment and removal.**
HeLa cells expressing EYFP-Parkin-C431S and mito-mCherry were treated with 10 µM CCCP for one hour followed by removal at T=60. Time lapse images display mutant Parkin recruitment to the mitochondrial membrane followed by minimal retention after removal of CCCP as compared to wildtype.

## Discussion

- The PINK1:Parkin mitophagy pathway is a quality control process in healthy cells, and mutations in genes encoding essential mitophagy proteins can be pathogenic. **Figure 1** displays notable active sites and functional domains of the PINK1 and Parkin proteins**. Figure 2** illustrates the relationship between PINK1, Parkin, and ppUb chains. Parkin is an E3 ubiquitin ligase that works in tandem with PINK1 to create phospho-polyubiquitin chains (ppUb) to mark mitochondria for degradation when depolarized.
- The purpose of this project was to evaluate the effects of the C431S mutation on the behavior of the Parkin protein hypothesized in **Figure 6** by observing Parkin accumulation and loss after depolarization. In this way, we can study the role of ppUb chains in Parkin retention following repolarization.
- The mutant was generated by 2-step PCR mutagenesis technique and verified through DNA sequencing as shown in **Figure 3.**.
- HeLa cells were transfected with either wildtype or mutant Parkin in combination with mitochondrial marker mito-mCherry to analyze kinetics under reversible depolarization. The timeseries was taken on a confocal microscope, 24 to 48 hours post-transfection. Cells were treated with 10 µM CCCP followed by removal at 60 minutes. Imaging was performed every three minutes for the duration of the experiment and analyzed using PCC (**Figure 5**).
- As expected, for HeLa cells expressing EYFP-Parkin, colocalization of Parkin with mito-mCherry increased rapidly during the initial 60 minute depolarization phase and decreased slowly over the following repolarization phase (**Figure 6**). While colocalization of the mutant Parkin protein with a mitochondrial marker appeared to decrease faster than the wildtype protein, these differences were small and the differences in mitochondrial Parkin levels at the start of the repolarization phase in the experiment made the comparison difficult. Future experiments could circumvent this particular issue by utilizing Parkin-R275W (**Figure 1**), which exhibits decreased E3 ligase activity but accumulates to similar levels as wildtype Parkin at depolarized mitochondria Parkin (Ordureau 2014).

## References

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