MEASURING THE RESPONSES OF THE MITOPHAGY PATHWAY TO TRANSIENT MITOCHONDRIAL STRESS AT THE LEVEL OF POLYUBIQUITIN AND PHOSPHO-POLYUBIQUITIN CHAIN FORMATION

by

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ABSTRACT

The PINK1:Parkin mitophagy pathway is a mitochondrial quality control system that functions to maintain the health and integrity of mitochondrial networks in somatic cells. Parkin proteins assemble polyubiquitin (pUb) chains on the surface of irretrievably damaged, depolarized mitochondria, and these chains are phosphorylated by PINK1 to create phospho-pUb (ppUb), a unique mitochondrial destruction signal. Here, we investigate the ability of the PINK1:Parkin pathway to discriminate between different levels and durations of mitochondrial stress by using biochemical techniques to study the recruitment, assembly, and persistence of PINK1 proteins, pUb, and ppUb to mitochondria under conditions of complete and transient mitochondrial depolarization.

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LIST OF ABBREVIATIONS

PD – Parkinson's disease

SNc – *substantia nigra pars compacta*

ROS – reactive oxygen species

MMP – mitochondrial membrane potential

IMM – inner mitochondrial membrane

OMM – outer mitochondrial membrane

TOM/TIM – translocase of the outer membrane/translocase of the inner membrane

PINK1 – PTEN-induced kinase 1

MPP – mitochondrial processing peptidase

Ub – ubiquitin

pUb – polyubiquitin

ppUb – phospho-polyubiquitin

OPTN – optineurin

NDP52 – nuclear dot protein 52

USP30 – ubiquitin carboxyl terminal hydrolase 30

CCCP – carbonyl cyanide m-chlorophenylhydrazone

I. INTRODUCTION

1.1 Parkinson's Disease

1.1.1 Background

Parkinson's Disease (PD) is a common neurodegenerative disorder, characterized by the presence of Lewy bodies, proteinaceous aggregates within neurons, and by the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc), a region of the midbrain largely associated with body movement and reward. An estimated 6.3 million people worldwide suffer from PD, and while the highest frequency of diagnoses is amongst those over 50 years old, early-onset familial forms of PD account for nearly 10% of all PD cases (Thomas and Beal, 2007). Although progress has been made toward achieving a fuller understanding of the etiology of PD, there is still much to learn regarding the molecular mechanisms underlying the disease. Recent PD research has implicated the dysfunction of two regulatory genes of mitophagy, *PINK1* (PINK1) and *PRKN* (Parkin), in genetic forms of PD (Narendra *et al.*, 2012). As a result of these findings, scientists have begun to focus their efforts on characterizing the mechanisms behind mitophagy – the selective autophagic degradation of dysfunctional mitochondria (Ding and Yin, 2012).

1.1.2 Pathology of Parkinson's disease

PD is pathologically distinguished from other types of Lewy body-associated neurodegenerative diseases by the combined presence of Lewy bodies and loss of dopaminergic neurons in the SNc. Characteristic indications of PD include the motor

symptoms of tremor, bradykinesia, and postural instability and the non-motor symptoms of hallucinations, depression, and dementia.

The primary component of Lewy bodies is α-synuclein, a protein which is thought to have a role in the normal release of dopamine at synapses in the SNc (Takeda *et al.,* 1998, Yavich *et al*., 2004). In people with sporadic PD or a genetic mutation in *SNCA*, the gene encoding for α-synuclein, abnormal levels of the protein have been implicated in neuronal toxicity (Polymeropoulos *et al.,* 1997, Conway *et al.,* 2000, reviewed in Wong and Krainc, 2017).

While the causes of dopaminergic neuron loss in PD patients remains elusive, abnormal mitochondrial morphology observed in the neurons of sporadic PD patients has led to the implication of mitochondrial dysfunction in the disease (reviewed in Henchcliffe and Beal, 2008). Dysfunctional mitochondria can ultimately lead to the accumulation of toxic reactive oxygen species (ROS), a normal byproduct of mitochondrial respiration and aging that heightens risk of increased mitochondrial membrane permeabilization leading to apoptosis (Aon *et al*., 2003). In those with genetic mutations in the PINK1 and Parkin genes, a defective mitophagy pathway is credited with the hallmark loss of dopaminergic neurons seen in heritable Parkinson's disease patients (Valente *et al.,* 2004, Zhang *et al.,* 2000). If the cell's normal mitophagy mechanisms are disrupted, defective mitochondria accumulate, ultimately leading to cell death. In undamaged cells, the mitophagy pathway exists as a mitochondrial quality control mechanism to prevent the accumulation of dysfunctional mitochondria, preserving cellular integrity (Lemasters, 2005).

1.1.3 Genetic factors influencing Parkinson's disease

To date, over a dozen genetic loci have been implicated in the onset of PD, though only six genes have been found capable of inducing PD monogenically (Klein and Westenberger, 2012). These monogenic mutations comprise about 30% of all familial PD cases and an estimated 5% of sporadic cases. Among the identified monogenic forms of PD, only the genes encoding for the proteins α-synuclein (*SNCA*) and leucine-rich repeat kinase 2 (*LRRK2*) exhibit an autosomal dominant inheritance pattern, with the remaining four (*PINK1, PRKN, DJ-1,* and *ATP13A2*) exhibiting autosomal recessive inheritance. The two most common of these recessive genes mutated in familial PD cases are those encoding PINK1 and Parkin. Studies by Greene *et al.* (2003) and Yang *et al.* (2006) show that PINK1 and Parkin-deficient *Drosophila melanogaster* populations demonstrate muscle and neuron degeneration, two hallmark consequences of PD. These studies suggest that loss of proteins responsible for mitochondrial quality control result in a buildup of damaged or defective mitochondria, and eventually a loss of dopaminergic neurons, leading to PD**.**

1.2 Mitophagy

1.2.1 Overview

Mitophagy is a mitochondria-specific form of autophagy that facilitates the removal of damaged or unwanted mitochondria. Mitophagy is a normal quality control response, generally triggered by agents such as ROS that damage mitochondria, which serves to protect cellular integrity by preventing the propagation of damage throughout a cell's mitochondrial networks and by preventing the release of pro-apoptotic factors from

the mitochondria (Kim *et al.,* 2007, Redman *et al.,* 2016). Damage to mitochondria can cause a reduction in mitochondrial membrane potential (MMP) – the difference in charge across the inner mitochondrial membrane (IMM). Disruption of MMP induces the recruitment of PTEN-induced putative kinase 1 (PINK1), a protein kinase, to the outer mitochondrial membrane (OMM; Jin *et al.,* 2010). PINK1 accumulates on the OMM and subsequently recruits, phosphorylates, and activates Parkin, a ubiquitin (Ub) ligase. From there, PINK1 and Parkin work together to attach and phosphorylate polyubiquitin (pUb) chains to OMM proteins to generate phospho-polyubiquitin (ppUb) chains – a unique marker of damaged mitochondria (Kane *et al.,* 2014). Establishment of ppUb chains at the OMM leads to the recruitment of autophagy receptors, such as optineurin (OPTN) and nuclear dot protein 52 (NDP52), which facilitate the assembly of a phagophore membrane around labelled mitochondria, isolating only these mitochondria from the rest of the cell prior to degradation upon fusion with lysosomes (Figure 1).

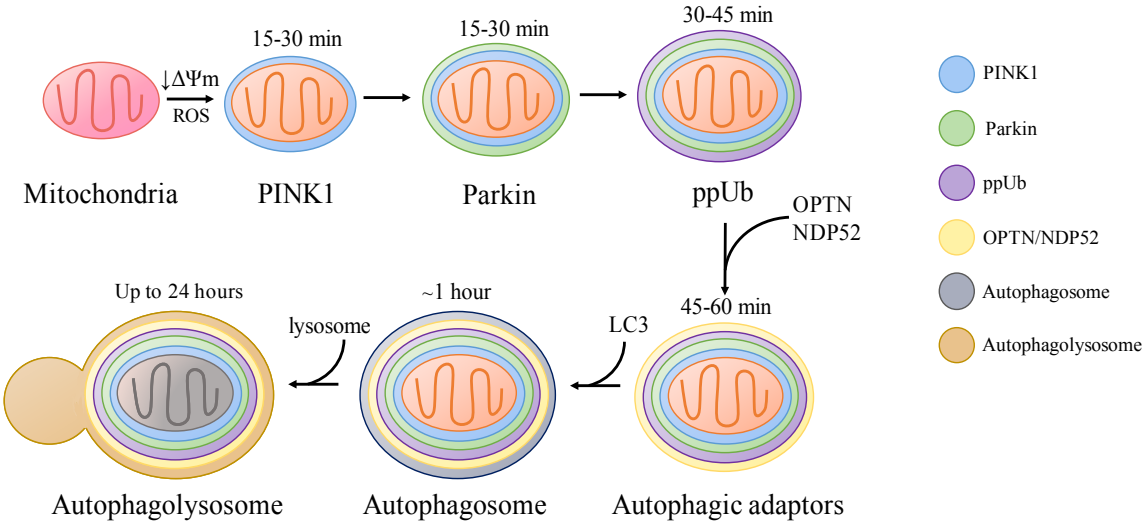


Figure 1: The timing of mitophagic and autophagic regulator recruitment. Upon mitochondrial depolarization, PINK1 and, subsequently, Parkin are recruited within 15-30 minutes, and ppUb chains are conjugated within 15-30 minutes after PINK1 and Parkin recruitment. Autophagic adaptors are then recruited and bind to the ppUb chains, leading to LC3 recruitment and autophagosome formation around 1 hour after initial depolarization. Finally, phagosome fusion with the lysosome and mitochondrial degradation requires up to 24 hours to complete.

1.2.2 The PINK1:Parkin pathway

PINK1 is a 64-kDa nuclear-encoded mitochondrial serine/threonine protein kinase, equipped with a mitochondrial targeting sequence at its N-terminus (Figure 2). It is constitutively imported through the OMM by the translocase of the outer membrane and translocase of the inner membrane (TOM/TIM) complexes at polarized, healthy mitochondria. Upon association with TIM, PINK1 is rapidly cleaved by the mitochondrial processing peptidase (MPP) and the transmembrane protease PARL into 60- and 52-kDa fragments, respectively, and exported to the cytosol for proteosomal degradation (Jin *et al.,* 2010). Matsuda *et al.* (2010) demonstrated that upon CCCP wash-out and complete repolarization of the mitochondria, PINK1 is quickly cleared from the

mitochondria and degraded to near-undetectable levels. This occurs through sequential processing in the mitochondrial matrix by MPP to a 60-kDa form, followed by further cleavage in the IMM by the protease PARL to form a mature, 52-kDa PINK1 form after import through the TIM complex (Jin *et al.,* 2010, Greene *et al.,* 2012). There remains some disagreement on the precise PINK1 degradation mechanism upon PINK1 cleavage by MPP and PARL: the 52-kDa mature fraction of PINK1 remaining in the TOM transporter either dissociates from the OMM to the cytosol, where it is degraded through N-end rule pathway, in which case it is then taken up, polyubiquitinated, and degraded (Yamano and Youle, 2013), or it follows a yet undefined mechanism of polyubiquitination and degradation (Liu *et al.,* 2017). While there exists some dispute over the precise degradation signal of mature PINK1, the current consensus is that it occurs via the proteasome in the cytosol.

However, when normal MMP is lost, often as a result of damage, though the import of proteins through the OMM via the TOM transporter remains unrestricted, import into the TIM complex is inhibited. This essentially causes PINK1 to become "stuck" in the OMM, with the C-terminal kinase domain exposed on the OMM, leading to an accumulation of PINK1 there (Youle and Bliek, 2012). The accumulated PINK1 recruits and subsequently activates Parkin, a cytosolic RING-Between-RING E3-ubiquitin ligase capable of polyubiquitinating mitochondrial substrate proteins on the OMM, once activated by PINK1-dependent phosphorylation at Ser65 on the Parkin UBL-domain (Kondapalli, 2012; Figure 2). Kazlauskaite *et al.* (2015) showed that once recruited to the OMM, Parkin is primed for PINK1-mediated phosphorylation of its UBL domain by binding to free phospho-monoubiquitin generated by PINK1. This action

causes a conformational change at the UBL domain, opening Parkin's conformation to expose the UBL's Ser65, which is then phosphorylated by PINK1. These conformational changes and activation allow Parkin to rapidly nonspecifically conjugate pUb chains to outer mitochondrial membrane proteins (Narendra *et al.,* 2008; Sarraf *et al.,* 2013, Kazlauskaite *et al.*, 2015). These chains are further modified by PINK1 through phosphorylation of Ser65 in a similar manner to Parkin, creating ppUb chains (Shiba-Fukushima *et al.,* 2014; Figure 3). Although it is well established that both PINK1 and Parkin are required for the synthesis of ppUb chains, the kinetics of mitochondrial accumulation and loss of PINK1, Parkin, and pUb chains in response to mitochondrial depolarization and repolarization has not been fully characterized. This information would enable us to better understand how the PINK1:Parkin mitophagy pathway might respond to physiological and time-varying stresses to provide new insights into how the pathway might filter or integrate different types of damage signals.

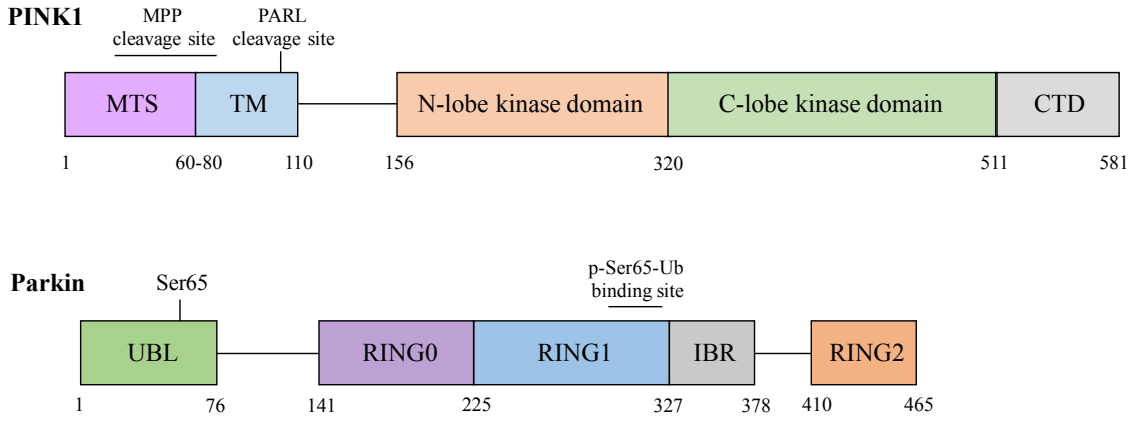


Figure 2: PINK1 and Parkin domain structures. PINK1 contains a mitochondrial targeting sequence (MTS) on its N-terminus, along with a transmembrane (TM) domain, and a C-terminal domain (CTD). MPP cleaves PINK1 on its MTS, and PARL cleaves at its TM. Parkin, an E3 ubiquitin ligase, contains really interesting new gene 0 (RING0), really interesting new gene 1 (RING1), in-between-ring (IBR), and really interesting new gene 2 (RING2) domains. The RING1 domain contains a phospho-Ser65-Ub binding site that, upon binding with a phosphorylated monoubiquitin molecule, causes Parkin to undergo a conformational change, opening its UBL domain. Parkin's UBL domain contains a Ser65 binding site that is phosphorylated by PINK1 to activate its Ub ligase ability.

1.2.3 Ubiquitin, polyubiquitin, and phospho-polyubiquitin

Ubiquitin is a small (~8 kDa) polypeptide that has many functions in eukaryotic organisms, including the targeting of proteins for degradation through the ubiquitin-proteasome system (UPS), and can be covalently conjugated as pUb chains in a variety of ways (reviewed in Clague *et al*., 2010). Ubiquitination plays a role in various mitochondrial processes including mitochondrial fission and fusion as well as mitophagy (Karbowski *et al.*, 2004). Within the context of mitophagy, ubiquitination of proteins on the OMM serves to tag damaged mitochondria for elimination and as a platform for the recruitment of Parkin and autophagy receptors (reviewed in Harper *et al.,* 2018).

Within minutes of Parkin recruitment to the mitochondria by PINK1, it begins nonspecifically mono- and poly-ubiquitinating substrates on the OMM (Sarraf *et al.*, 2013). Though several types of lysine-linked Ub chains exist, and *in vitro* experiments have showed that Parkin can direct the formation of pUb chains with four specific linkages (K6, K11, K48, and K63), two are of particular interest as they have been shown to play a definitive role in the clearance of defective mitochondria and their associated membrane proteins: the K48 and K63 chain types. While K48 and K63 pUb both have integral roles in mitophagy, these roles may differ. K48 pUb has long been known to signal for proteosomal degradation (Finley *et al.,* 1994), which is consistent with its assumed role in the mitophagy pathway; Parkin-conjugated K48 chains serve as a signal for degradation of OMM proteins (Chan *et al.,* 2011). The roles of K63 are widely studied and vary greatly throughout the cell, such as those involved in DNA damage repair and protein kinase activation, as is seen throughout the NF-κB pathway (Hofmann and Pickart, 1999, Pickart and Fushman, 2004, Krappmann and Scheidereit, 2005), however, its role in the mitophagy pathway has remained more elusive until recently (Kane *et al.,* 2014).

pUb chain assembly on OMM proteins is soon followed by phosphorylation of only K48 and K63 chains by PINK1 at Ser65 within individual Ub moieties (Ordureau *et al.,* 2014), a structurally similar site to Ser65 found on Parkin's UBL domain (Ordureau *et al.,* 2014); in fact, it has been shown that around 20% of all mitochondrial ubiquitin molecules are phosphorylated upon mitochondrial depolarization with the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP; Ordureau *et al.,* 2015). Once formed, the ppUb chains act as new docking sites for active Parkin, creating a cascade or

positive-feedback loop, and lead to the conjugation of yet more phospho-polyubiquitin chains to the OMM at damaged mitochondria (Ordureau *et al.,* 2014; Figure 3).

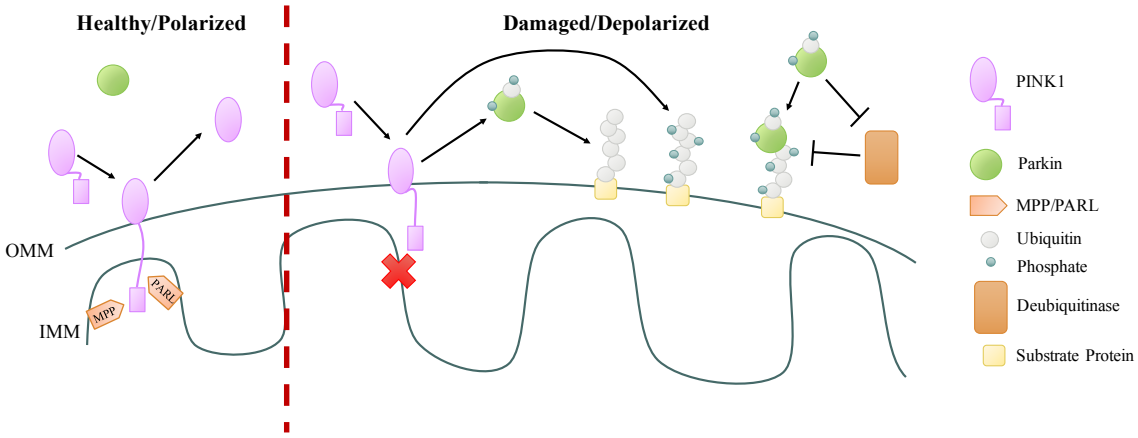


Figure 3: Detailed depiction of the mitophagy pathway. In healthy, polarized mitochondria, PINK1 associates with TOM complexes at the OMM by its mitochondrial targeting sequence, where it is imported by the TOM:TIM transporters and cleaved by MPP in the intermembrane space and by PARL within the IMM. PINK1 then dissociates from the OMM and is degraded in the cytosol. In damaged, depolarized mitochondria, PINK1 associates with TOM but cannot enter the TIM transporter, causing it to remain associated with TOM, with the majority of PINK1 exposed on the OMM, facing the cytosol. PINK1 then recruits, phosphorylates, and activates Parkin, and together they conjugate ppUb chains on OMM substrate proteins. Active Parkin can utilize ppUb chains as a receptor, anchoring these proteins on the OMM. While deubiquitinases such as USP30 aim to disassemble pUb and ppUb chain on the OMM, active Parkin actively opposes these enzymes.

1.2.5 Autophagosome formation and mitochondrial degradation

To complete mitophagy, ppUb chains recruit and serve as docking sites for several autophagy receptors, including OPTN and NDP52, at the OMM (Lazarou *et al.*, 2015, Heo *et al.,* 2015). These autophagy receptors contain an LC3-interacting region (LIR), allowing them to bind to LC3, the primary protein component of the phagophore membrane, as well as ppUb chains (Lazarou *et al.,* 2015, Shpilka *et al.,* 2011). While

OPTN and NDP52 have been shown to be similar in structure and are recruited with similar kinetics following mitochondrial depolarization, their functions vary slightly. OPTN encircles mitochondria undergoing fragmentation while NDP52 binds at the interface between mitochondrial fragments as they cluster at the nuclear periphery prior to being degraded en masse after fusion with lysosomes (Moore and Holzbaur, 2016). In addition, OPTN, but not NDP52, recruitment is an essential and rate-limiting step in mitochondrial autophagosome formation (Moore and Holzbaur, 2016). Recent evidence suggests that LC3 recruited by ppUb can itself recruit OPTN and NDP52 independently of further Ub chain conjugation, amplifying the rate of autophagosome recruitment and, thus, mitophagy (Padman *et al.,* 2019). The phagophore membrane develops around the damaged mitochondria to form an autophagosome that later fuses with lysosomes in a process that can take hours to complete (Bampton *et al*., 2005; Figure 1).

1.3 Factors influencing mitophagy

1.3.1 Deubiquitinases and PTEN-L

Recent study of the mitophagy pathway has revealed that the process of pUb assembly is actively opposed by deubiquitinating enzymes, including ubiquitin carboxyl-terminal hydrolase 30 (USP30), which inhibits the mitophagy mechanism by cleaving Ub chains on the OMM (Bingol *et al.,* 2014). While pUb and ppUb chains are both removed from the OMM by USP30, the removal of ppUb is thought to be less efficient than that of pUb (Wauer *et al.,* 2014, Bingol and Sheng, 2016, Huguenin-Dezot *et al.,* 2016). While USP30 has been shown to oppose mitophagy, Bingol *et al.* (2014) show that Parkin negatively regulates USP30 by targeting it for degradation.

A recent study by Wang *et al*. (2018) demonstrated evidence for a novel phosphatase that directly dephosphorylates ppUb during mitophagy. Because USP30 is so inefficient at disassembling ppUb chains, the phosphatase, PTEN-L, could provide a mechanism by which ppUb could be readily converted back to pUb and rapidly removed by USP30 and other mitochondrial deubiquitinases.

1.3.2 Feedback loops

The protein-protein interactions involved in the mitophagy pathway create a network of interconnected feedback loops or network motifs (Mangan and Alon, 2003), which include both positive feedback and coherent feedforward loops. These network motifs were originally developed as a means to describe genetic circuits but can also be used to classify cellular signaling networks, such as those found in the mitophagy pathway. While positive feedback loops serve as amplifiers of a specific process (i.e. the amplification of Parkin recruitment and ppUb deposition through the Parkin:ppUb feedforward loop**),** coherent feedforward loops provide networks with the ability to ignore transient signals and to only provide a response to persistent stimulation (Shen-Orr *et al.*, 2002).

Within the context of the PINK1:Parkin mitophagy pathway, the recruitment of Parkin to the OMM by PINK1 and subsequent assembly of ppUb chains by these proteins working in tandem constitutes a coherent feed-forward loop (Figure 4A). The binding of Parkin to ppUb chains, which it plays a role in forming, constitutes a positive-feedback loop (Figure 4B). The relationship between, USP30, Parkin, and ppUb can also be described as a coherent feed-forward loop (Figure 4C). When these network motifs are

combined, they constitute a network featuring two interlocking coherent feedforward loops and a positive feedback loop (Figure 4D). Although predictions of protein responses to stress can be made based on these network motifs, it is impossible to intuit these responses in transient and repeated stress conditions due to the complexity of the pathway when taken as a whole. In fact, we would expect the pathway to exhibit non-linear behaviors as a result of this complexity.

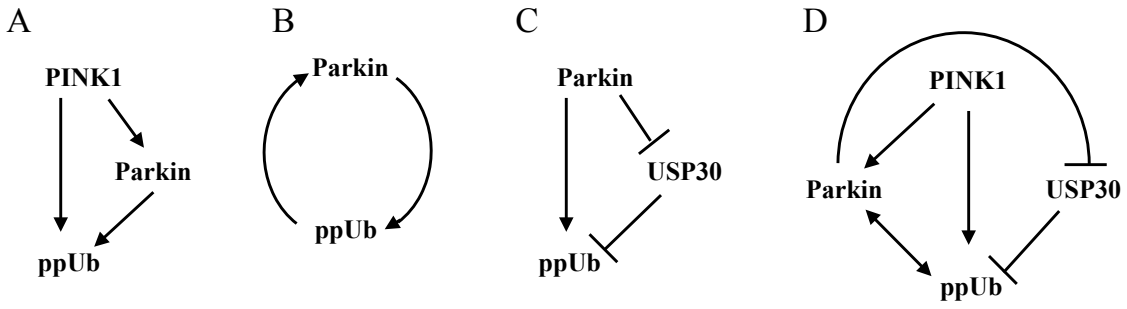


Figure 4: Network motifs in the PINK1:Parkin pathway. (A) PINK1 activates Parkin, and, together, PINK1 and Parkin create ppUb chains on the OMM in a coherent feed-forward loop. (B) Parkin and ppUb associate on the OMM, constituting a positive feedback loop. (C) Parkin actively inhibits USP30, which promotes ppUb disassembly, in a coherent feed-forward loop. (D) The combined network diagram.

1.4 Study Objectives and Hypotheses

Mitochondria have been shown to exhibit membrane potential fluctuations *in vivo* in a variety of cell types. For example, Lisa *et al.* (1995) demonstrated that cardiac myocytes experience synchronous oscillations of MMP during oxidative stress; neuronal cells (Buckman and Reynolds, 2001) and smooth muscle cells (Chalmers and McCarron, 2008) have been shown to experience similar fluctuations *in vivo* even under normal conditions. As the current literature that explores the response of the mitophagy pathway

to changes in MMP typically uses treatments that cause irreversible mitochondrial damage or complete and persistent loss of MMP to characterize the response of the pathway to stress, we believe that by mimicking normal membrane fluctuations in our investigation into the pathway, we can more accurately investigate how the pathway would respond to stresses as they might occur *in vivo.*

We anticipated that transient and repeated periods of mitochondrial stress would allow for a more accurate depiction of how the cell interprets 'real-life' stresses as they may occur in the beginning stages of PD or as a product of the aging process. To achieve this goal, we first measured PINK1 levels in all cells exposed to different CCCP concentrations to induce partial or complete loss of MMP as well as subjecting cells to repeated rounds of de- and repolarization to determine the recruitment and persistence of the protein, as it is a necessary contributor to mitophagy in the recruitment and activation of Parkin and ppUb. Next, we aimed to assess the recruitment and persistence of two polyubiquitin chain types – K48 and K63 – under transient conditions. Finally, we evaluated ppUb assembly and persistence under continuous and transient loss of MMP in hopes of understanding the pathway's response to these types of insults as a whole. We hoped to use the results of this study to create a comprehensive understanding of how the mitophagy pathway responds to more authentic levels and durations of stress than has previously been reported. Although the biochemistry of the enzymes that add and remove ppUb from mitochondria has been well characterized, how their activities are balanced and regulated to appropriately respond to different levels of mitochondrial stress and only eliminate those mitochondria that cannot be recovered is not well understood. Furthermore, it is unclear how this network, which is responsible for detecting and

responding to mitochondrial insults, interprets low-level or transient mitochondrial stresses.

Because our data suggest that the mitophagy pathway is capable of ignoring pulses of minor insults at the PINK1 level, this raises interesting questions about how the pathway interprets and responds to varying levels and durations of stress at the level of and downstream of PINK1. As PINK1 has been shown to quickly dissociate from the OMM upon removal of stress and ppUb chains are thought to be relatively resistant to degradation by USP30, we hypothesized that K48- and K63-ppUb chains would act as a persistent marker of prior mitochondrial damage, possibly priming the mitochondria for mitophagy in response to repeated damage. We hypothesized that these chains would remain on OMM proteins even after repolarization and PINK1 dissociation from the OMM, while pUb chains would be more short-lived.

Indeed, our findings from this study suggest that while PINK1 rapidly dissociates from the OMM on a timescale of minutes, ppUb and K63 chains remain for far longer, taking in excess of an hour to remove, supporting our hypothesis that these chains may serve as a type of cellular “memory” in cells undergoing repeated mitochondrial insults over time.

II. MATERIALS AND METHODS

2.1 Mammalian cell culture

HeLa cells, a human cervical adenocarcinoma cell line, were obtained from the American Type Culture Collection (ATCC), and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and sodium pyruvate and supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin and Streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO_2. Cells were seeded at a density of $5x10^6$ in 60 mm dishes for western blot analysis or $1x10^5$ in 35 mm glass-bottom dishes (Cellvis) for immunofluorescence analysis.

As HeLa cells are effectively Parkin-null and do not express the endogenous protein, cells used in ppUb western blot and K48, K63 immunofluorescence experiments were transfected using the pEYFP-Parkin construct produced in-house by Jonathon Logan Bowling to reconstitute the PINK1:Parkin pathway.

2.2 Transfection Procedure

Transfection of pEYFP-Parkin into HeLa cells was performed using 1 μg/mL polyethyleneimine (PEI) transfection reagent (Polysciences). PEI was made in sterile water by heating to 56°C to dissolve the solid PEI in water, the pH was adjusted to pH 7.2 with HCl, and filter sterilizing using a 0.22 μm filter. A 1:3 (μg: μL) ratio of plasmid DNA:PEI was diluted in serum-free media and allowed to incubate for 20 minutes at room temperature in order to form DNA:liposome complexes before adding to seeded cells. In 35 mm glass-bottom dishes, 1 μg plasmid DNA and 3 μL PEI were added to

each dish and incubated in serum-free media for 4-5 hours. For 60 mm dishes, 4 μg plasmid DNA and 12 μL PEI reagent were added to each dish and incubated for 4-5 hours. In all cases, residual PEI was removed by washing the cells in 37°C PBS before replacing with fresh culture medium. Typically, cells were analyzed 24 hours post-transfection.

2.3 Immunoblotting

After disruption of cellular MMP using carbonyl cyanide m-chlorophenyl hydrazine (CCCP; ThermoFisher) at the concentrations and times indicated in figure legends, cells utilized in PINK1 experiments were lysed in RIPA buffer (1% TritonX-100, 10% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, and 1 mM EDTA) containing 1X protease inhibitor cocktail (Sigma Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich), and cells used in ppUb experiments were lysed directly in 2X Laemmli buffer buffer (0.5 M Tris-HCL, pH 6.8, 20% (w/v) glycerol, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 2.5% (w/v) beta-mercaptoethanol) to preserve polyubiquitin chains. Harvested cells were vigorously vortexed for 30 seconds every five minutes for 30 minutes and centrifuged at 15,000 x g at 4°C in a refrigerated bench-top microcentrifuge for 15 minutes to pellet cell debris and unlysed cells. The protein concentration of RIPA lysates was determined by using a bicinchoninic acid (BCA) assay kit (Pierce) and normalized to the same concentrations by dilution in RIPA buffer. Laemmli buffer was added to normalized samples at a final concentration of 1X, and samples were boiled at 90°C for

10 minutes to denature the proteins. Prepared samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to an Amersham™ Protran™ nitrocellulose membrane (GE Healthcare) using a Bio-Rad™ Trans-Blot SD Semi-Dry Transfer Cell for 55 minutes. Membranes were blocked in tris-buffered saline/0.2% tween-20 (TBS/T) containing 5% (w/v) nonfat milk powder for one hour and washed in TBS/T 3 times for 5 minutes each before incubation with the indicated primary antibodies overnight (~16 hours) at 4°C.

Primary antibodies used for western blot samples were as follows: β-actin (PA1-16889, ThermoFisher Scientific), Parkin (ab15954, Abcam), phospho-ubiquitin Ser65 (37642; Cell Signaling), PINK1 (D8G3, Cell Signaling). Anti-rabbit (sc-2313; Santa Cruz Biotechnology) or anti-mouse (sc-51612; Santa Cruz Biotechnology) HRP-conjugated secondary antibodies were used at a 1:5000 dilution in milk-TBS/T and incubated with membranes for at least one hour at room temperature. After secondary antibody incubation, membranes were washed in TBS/T 3 times for 5 minutes each. The blots were developed by incubation for ~1 minute with a 50:50 mix of enhanced chemiluminescence (ECL) detection reagents I (250 nM luminol, 90 mM pCoumaric acid, and 0.1 M tris, pH 8.5) and ECL reagent II (30% H_2O_2 in 0.1 M tris, pH 8.5). Blots were imaged using Image Lab™ Software on a ChemiDoc MP Imaging System (Bio-Rad). Integration times for image capture typically ranged between 30 seconds and 9 minutes, depending on protein concentration and the primary antibody used.

2.4 Immunofluorescence

EYFP-Parkin-transfected HeLa cells plated in 35 mm glass-bottom dishes were treated with 10 μM CCCP for the times indicated. They were then fixed in 3% paraformaldehyde at room temperature for 10 minutes and permeabilized with 0.25% Triton X-100 on ice for 5 minutes. Cells were then blocked at room temperature in 5% goat serum-phosphate buffered saline (PBS) for 30 minutes, with 2 washes in ice-cold PBS between each step. Cells were incubated with either anti-K48 (05-1307, Millipore Sigma) or anti-K63 polyubiquitin monoclonal antibodies (05-1308, Millipore Sigma) diluted to 1:500 in 5% goat serum-PBS overnight (~16 hours) at 4°C. Cells were then washed in PBS and incubated in AlexaFluor 647 anti-rabbit secondary antibody (ab150075, AbCam) for 30 minutes at room temperature. Cell nuclei were stained with DAPI using NucBlue Live Cell Stain (NucBlue™ Fixed Cell ReadyProbes™ Reagent, ThermoFisher Scientific) and imaged using a Zeiss LSM700 laser scanning confocal microscope.

2.5 Confocal Microscopy

Immunofluorescence assay samples were imaged using a Zeiss LSM700 laser scanning confocal microscope equipped with a Plan-Apochromat 63X magnification/1.40 numerical aperture oil immersion DIC objective lens (Carl Zeiss). AlexaFluor 647 was excited using a 639 nm laser, EYFP fluorescence was excited using a 488 nm laser, and DAPI fluorescence was excited using a 405 nm laser. The microscope was controlled using Zen software (Zeiss). The same software package was used to assess colocalization

of stained proteins by Pearson's Correlation Coefficient (PCC). All images used in figures were exported as 16-bit Zeiss .tif images.

2.6 Statistical Analysis

Densitometry of western blot images was performed using FIJI software (Schindelin *et al.,* 2012). Data are normalized to actin levels and are expressed as mean ± SEM.

Colocalization was assessed by PCC in Zen (Zeiss). Image threshold was set using the threshold value method set by Jaskolski *et al.* (2005), and colocalization between EYFP-Parkin and either K48 or K63 was measured by drawing an outline around the whole cell. Data were exported through Microsoft® Excel and are expressed as mean ± SEM.

All experiments have been performed in triplicate.

III. RESULTS

3.1 PINK1 is rapidly processed and cleared from fully repolarized mitochondria.

PINK1 stabilization at mitochondria is the crucial first step in the formation of OMM ppUb chains, the signaling molecules responsible for autophagy receptor recruitment to damaged or defective mitochondria (Shiba-Fukushima *et al.,* 2014, Lazarou *et al.,* 2015). It is well understood that full-length PINK1 is stabilized and accumulates at the OMM upon complete loss of MMP (Narendra *et al.*, 2010), an effect that is commonly studied *in vitro* upon cell treatment with the mitochondrial protonophore and inhibitor of oxidative phosphorylation, CCCP (Narendra *et al.,* 2008, Vives-Bauza *et al.,* 2009). Although it has been shown that PINK1 rapidly associates with the mitochondrial membrane during complete loss of MMP (Narendra *et al.*, 2010), these studies do not address the response of PINK1 to partial depolarization events and fluctuations in MMP, as might be expected in aging cells or cells experiencing long-term mitochondrial stress.

While the rapid loss of PINK1 from the mitochondria upon complete mitochondrial repolarization has been previously documented (Matsuda *et al.*, 2010), we aimed to characterize the protein's response to partial repolarization. Since rapid clearance of PINK1 is known to accompany complete recovery of MMP, it was hypothesized that partial PINK1 clearance would follow partial MMP recovery. Previous work in our lab has determined concentrations of CCCP appropriate for total, partial, and minimal mitochondrial membrane depolarization (10, 5, and 2.5 μM, respectively) using the mitochondrial membrane potential dye, Tetramethylrhodamine, methyl ester (TMRM). Using these previously-determined CCCP concentrations, we fully depolarized

and then repolarized the mitochondria to evaluate the responses of PINK1 in HeLa cells. This was performed by inducing complete loss of MMP in cells with one hour of treatment in 10 μM CCCP. After the first hour of treatment, cells were either further treated with 10 μM CCCP for another hour, media was washed out and replaced with fresh media containing 5 or 2.5 μM CCCP, or treatment media was replaced with media containing no CCCP (Figure 5A). While stabilization of full-length PINK1 resulted from complete, sustained loss of MMP (10 μM), CCCP concentrations associated with minimal and no membrane depolarization produced near-complete loss of full-length PINK1 in cells (Figure 5B, C). Although it was expected that the 5 μM CCCP treatment would result in a more robust clearance of full-length PINK1 than was observed (Figure 5B, C), overall this treatment closely resembled that seen in the cells that underwent continuous total mitochondrial depolarization; however between the three repeats of this experiment, the 5 μM CCCP treatment results varied greatly between each repeat (Figure 5B and Appendix A), suggesting that 5μM CCCP doses reduced TIM function close to the threshold level required for PINK1 import.

Together, these data suggest that even after partial repolarization, PINK1 is quickly cleared from the mitochondria. The return of PINK1 to basal levels within 30 minutes of total repolarization following a complete depolarization event was especially interesting and prompted more questions regarding the response of PINK1 to repeated mitochondrial insults.

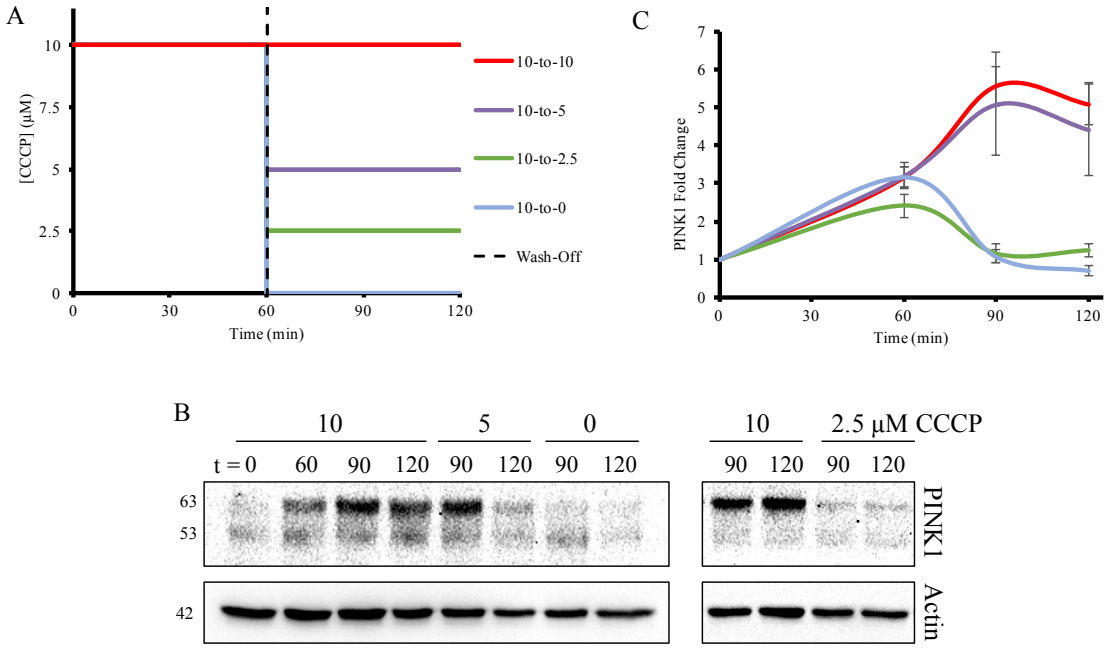


Figure 5: PINK1 rapidly degraded after partial and complete repolarization of mitochondria. HeLa cells were treated with (A) the above concentrations of CCCP over two hours. (B) A representative image of a western blot from samples that were harvested and immunoblotted for PINK1 and actin as a loading control, and (C) western blot data over three replicates was quantified using ImageJ software (NIH; for other western blots from separate biological repeats of this experiment, see Appendix A).

3.2 PINK1 Accumulation Upon Consecutive Pulses of Mitochondrial Stress.

As we previously established that PINK1 is rapidly degraded upon partial or complete repolarization of the mitochondria, we next aimed to characterize the response of PINK1 to consecutive insults after the return of PINK1 to basal levels in the cell. To determine whether prior insults could influence the response of PINK1 to subsequent stress, we implemented a pulsing regime based on the results from the previous experiment (Figure 5). Since we knew that PINK1 levels rise steadily and peak at ~60 minutes in cells exposed to 10 μM CCCP, and that PINK1 levels decrease rapidly, returning to basal levels within 30 minutes of CCCP removal, we chose a '60 minute on, 40 minute off' scheme to allow ample time for PINK1 clearance prior to subsequent rounds of de- and repolarization (Figure 6A).

This two-pulse treatment regime resulted in the expected rise in PINK1 levels after 60 minutes of depolarization, followed by the return of PINK1 to basal levels after 40 minutes of total membrane repolarization through CCCP wash-out. The second pulse of CCCP led to a robust increase in PINK1 protein concentration, followed by loss to undetectable levels (Figure 6B, C). We believe that the decrease in PINK1 to near-undetectable levels may be explained by a period of rapid import of stabilized PINK1 into the IMM following repolarization and reactivation of TIM transporters

As there was found to be a small but statistically significant difference between the levels of PINK1 at the 'peaks' seen in the western blot (Figure 6B, C), this result demonstrated that PINK1 can accumulate on the OMM in higher concentrations after an initial mitochondrial insult – a response that is contrary to previous live cell data from the Nelson lab (unpublished data). We anticipate this unexpected result could be caused by

the expression of endogenous PINK1 in western blot experiments and exogenous PINK1-EGFP constructs in live cell experiments.

In addition to these PINK1 experiments, previous data show that Parkin, when exposed to similar repeated mitochondrial membrane depolarization events, accumulates over time and is slow to dissociate from the mitochondria (unpublished data from the Nelson lab). As it has been shown that Parkin utilizes ppUb chains as receptors, anchoring the protein on the mitochondria (Ordureau *et al.,* 2014), this led to our investigation of the downstream product of PINK1 and Parkin, ppUb.

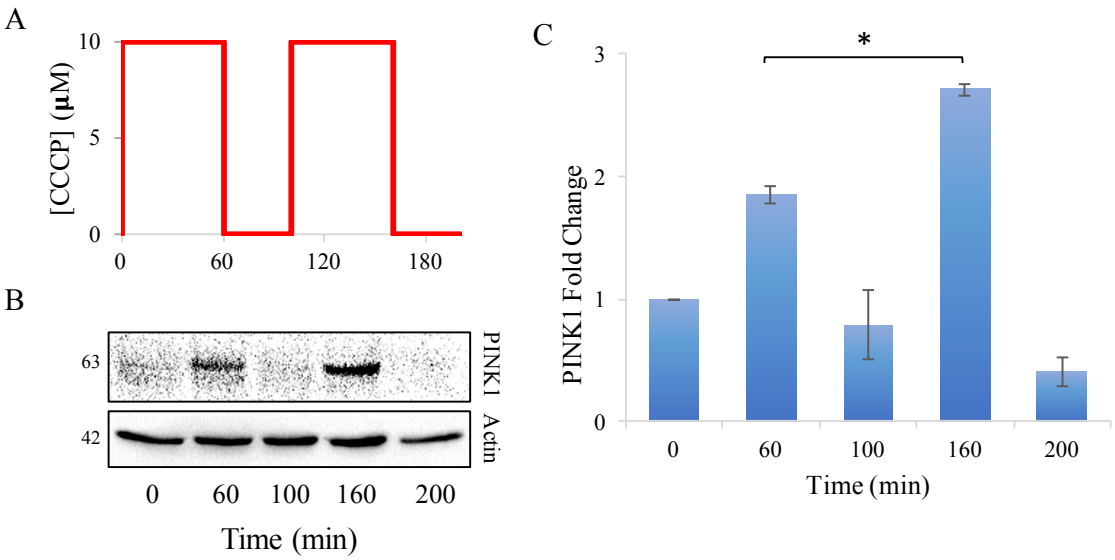


Figure 6: PINK1 accumulation upon repeated mitochondrial insults. HeLa cells were treated (A) with 60 minute pulses of 10 μM CCCP every 100 minutes, and (B) harvested at the indicated time points and immunoblotted for PINK1 and actin as a loading control. (C) PINK1 levels were quantified by densitometry using western blot data over 3 experiments using ImageJ software (NIH). Error bars represent S.E. Statistical significance is represented as follows: $p < 0.05$ (one-way ANOVA and Tukey's post-hoc test), N = 3. Full statistics for these data can be found in Appendix B.

3.3 Phospho-polyubiquitin Chains as a Lasting Marker of Mitochondrial Depolarization.

Phospho-polyubiquitin chains, which are first conjugated as polyubiquitin chains on OMM substrate proteins by Parkin and are subsequently phosphorylated at available Ser65 sites by PINK1, recruit autophagy receptors to the OMM that then trigger autophagosome formation around damaged mitochondria (Ordureau *et al.,* 2014, Lazarou *et al.,* 2015). While this seems straightforward, further investigation into ppUb chain types and functions has revealed that ubiquitin serves a much more complex role in the mitophagy pathway. Investigation into the number and variation of pUb chain types, as well as their potential for phosphorylation by PINK1, has revealed a complex system of pUb and ppUb chain formation and modification, contributing to a variety of roles for each of these chain types and their potential modifications (reviewed in Harper *et al.*, 2018). The complex interactions between pUb chains, the proteins recruited to them, and the damaged mitochondria to which they are conjugated have not been fully resolved.

Although the role of ppUb in the mitophagy pathway has been well-studied, there is still much to uncover about the kinetics of ppUb removal upon mitochondrial repolarization. Because ppUb is thought to be resistant to the effect of deubiquitinases, we proposed that ppUb would act as a marker of persistent mitochondrial damage, remaining on the OMM even after PINK1 dissociation from the mitochondrial membrane. Recent data from the Nelson lab suggests that Parkin remains associated with the mitochondria after PINK1 processing and removal; we believe that this is due to the tethering of Parkin to ppUb chains, and we expected ppUb to exhibit similar kinetics to Parkin in terms of its accumulation on mitochondrial depolarization and loss on

repolarization. To investigate the persistence of ppUb post-repolarization, HeLa cells transfected with EYFP-Parkin expression constructs were treated with 10 μM CCCP for one hour to fully depolarize the mitochondria. After the initial 60 minutes of treatment, all CCCP was washed out, and ppUb was examined at 30, 60, 120, and 300 minutes post wash-out by western blotting; we also examined ppUb under a continuous treatment with 10 μM CCCP at equivalent timepoints (Figure 7).

As expected, ppUb was detectable via western blot 60 minutes post CCCP wash-out in the transient stress experiment (Figure 7). Although ppUb levels steadily decreased upon CCCP wash-out, the lack of immediate total removal from the mitochondria, as was seen in our PINK1 experiments, suggests that ppUb chains may indeed serve as a type of persistent marker of mitochondrial depolarization events by remaining on the OMM after mitochondrial repolarization and PINK1 degradation. The continuous CCCP treatment experiments showed that ppUb concentrations remained high throughout the duration of the experiment.

After investigating the kinetics of ppUb loss after repolarization of the mitochondria, we next aimed to examine the persistence of K48 and K63 chains post-repolarization. These lysine-linked pUb chain types were an obvious next target in our studies of this pathway as they are both assembled at the OMM by Parkin and phosphorylated by PINK1 during mitophagy, becoming ppUb chains (Okatsu *et al.,* 2015). We believed that investigation into specific Ub chain linkage types would help elucidate the roles of the specific ppUb chains and further our knowledge of the kinetics behind their assembly and disassembly on the OMM.

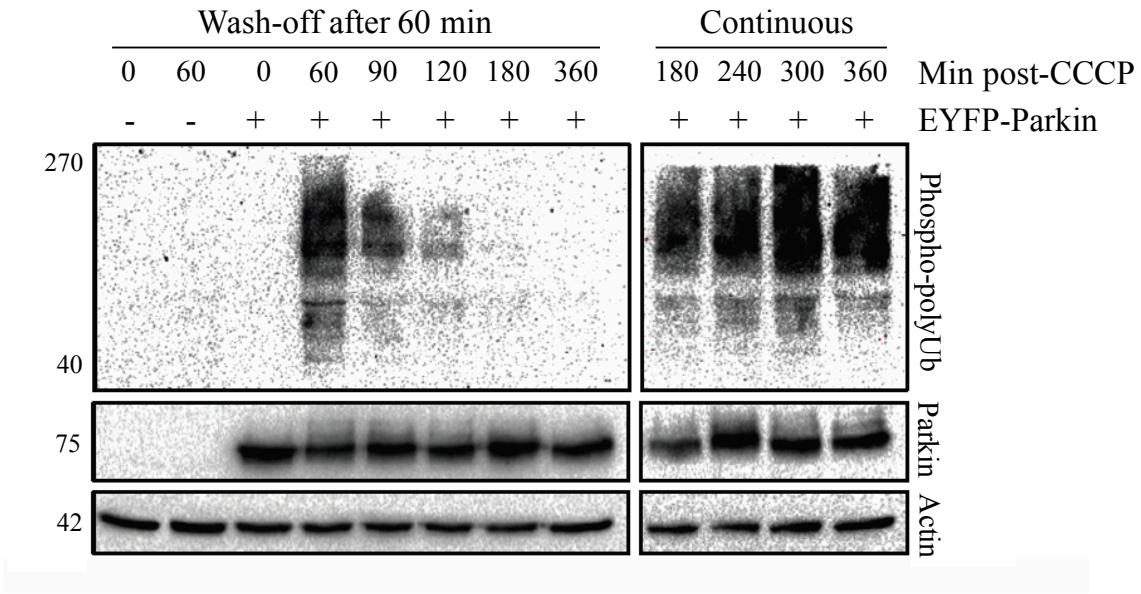


Figure 7: ppUb serves as a transient marker of MMP loss. Western blot analysis for phospho-polyubiquitin levels in EYFP-Parkin expressing HeLa cells treated with either a 60 minute pulse or continuously with 10 μM CCCP.

3.4 K48 and K63 Differ in Disassembly Rates from the Mitochondria Upon Removal of Mitochondrial Stress.

During mitophagy, Parkin-conjugated K48 and K63 pUb chains on OMM proteins can be phosphorylated by PINK1 on Ser65 sites throughout the chains. These ppUb chains are resistant to degradation by deubiquitinases and may remain on the mitochondrial membrane for at least one hour after mitochondrial repolarization (Figure 7). In other parts of the cell, however, unmodified K48 chains are known to be involved in proteasomal degradation (reviewed in Clague and Urbé, 2010), while K63 chains have been implicated in the regulation of several non-proteolytic processes throughout the cell, including membrane trafficking, DNA damage repair, and protein kinase activation (reviewed in Chen and Sun, 2009). Since the ppUb antibody used in our western blot analysis did not discriminate between phosphorylated K48 and K63 chain types and because Ser65-ppUb is unique to mitophagy, it was possible to assay for the protein using western blotting on whole cell lysates; however, as K48 and K63 polyubiquitination occurs throughout the cell continuously, it was essential to use a method that could allow us to separate ubiquitination occurring at the mitochondria from that occurring in other compartments of the cell. Thus, we aimed to observe the mitochondrial assembly and removal of each of the two chain types individually using immunofluorescence.

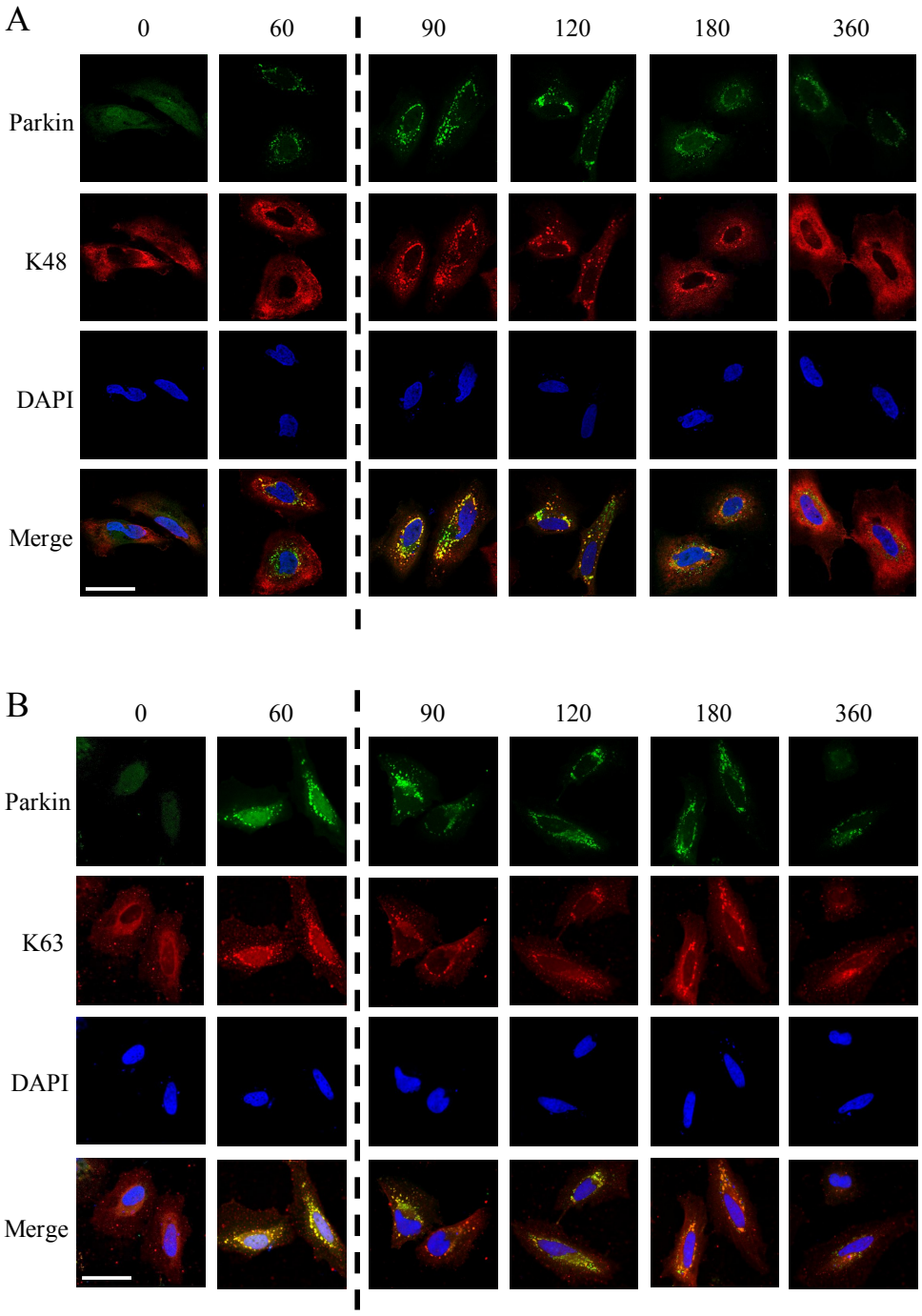
Assembly of K48 and K63 chains on the mitochondria was evaluated by measuring colocalization between each of the chain types and Parkin using antibodies specific for K48 or K63 chain types, regardless of whether they were phosphorylated. As it is well documented that Parkin associates with the mitochondria, utilizing ppUb chains as receptors, this measurement was sufficient to assess mitophagy-related co-association

of these molecules and the mitochondria. As with transient ppUb western blot experiments, HeLa cells transfected with EYFP-Parkin were transiently treated with 10 μM CCCP for one hour. After the initial hour of treatment, the cell media containing CCCP was washed off, and cells were evaluated at 30, 60, 120, and 300 minutes post-CCCP wash-out.

The presence of K48 and K63 chains on the mitochondria was measured by evaluating their colocalization with EYFP-labeled Parkin. The results of these experiments revealed that while K48 and K63 are both assembled on OMM proteins within 60 minutes of mitochondrial depolarization, they are both relatively slow to disassemble, especially when these data are considered in contrast with the PINK1 wash-out western blot experiments (Figures 6, 8). However, K48 chain colocalization with Parkin returned to near-basal levels within two hours of CCCP wash-out, while K63 levels failed to return to the baseline, remaining colocalized with Parkin for at least 300 minutes post-CCCP wash-out (Figure 8). We believe that the discrepancy in the rate of K48 and K63 loss post-repolarization may be due to the known differences in these two chain types. K48, being associated with UPS, would likely be short-lived before or after the removal of Ser65 phosphorylation, the precise function of which is unknown, while K63 would not.

The observation that K63 chains remain colocalized with Parkin hours after the removal of stress further supports our hypothesis that ppUb chains serve as a persistent marker of mitochondrial stress, priming mitochondria for a more rapid response to subsequent damage. To further assess the roles of K48 and K63 in the mitophagy pathway and to further confirm our hypothesis that K48 chains are removed from the

mitochondria more readily than K63 chains, we next aimed to characterize the responses of these proteins to continuous stress.



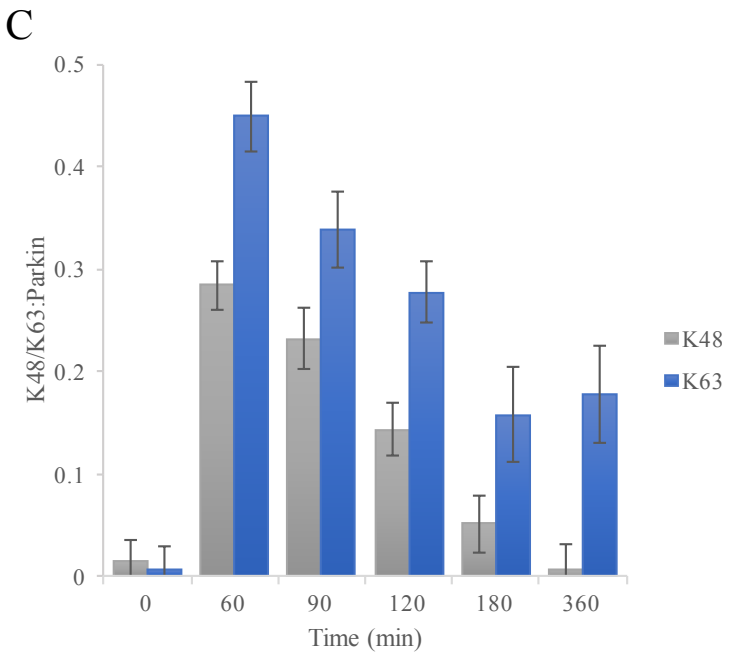


Figure 8: K48 and K63 polyubiquitin chains disassemble at different rates. Fluorescence microscopy images of HeLa cells expressing EYFP-Parkin (green), fixed at the indicated timepoints during and post-CCCP treatment and stained with NucBlue (blue) and either (A) K48 antibodies (red) to detect K48 polyubiquitin or (B) anti-K63 antibodies (red) to detect K63 polyubiquitin. Scale bars represent 50 μm. (C) The colocalization of EYFP-Parkin and K48 or K63 was assessed using Pearson's correlation coefficient (PCC) and graphed as the average PCC score. Data is from a minimum of 3 biological repeats with a minimum of 56 cells per condition. Error is represented as the S.E.

3.5 K48 is Removed from the Mitochondria More Readily than K63 During Continuous Mitochondrial Stress.

Since we found that K48:Parkin colocalization returns to the baseline much more quickly than that of K63 after transient depolarization, we speculated that during continuous mitochondrial stress, K48 chains would only remain on the OMM long enough to signal for degradation of the proteins to which they are attached. Under continuous stress events, it was hypothesized that K48 chain colocalization would decrease over time as these chains are degraded, but K63 chains concentrations would remain high over time as it can take hours to accomplish mitophagy (Bampton *et al*., 2005). Indeed, initial experiments have revealed that after 180 minutes of continuous treatment with 10 μM CCCP, K48 chains are much less colocalized with Parkin at the mitochondria than K63 chains (Figure 9). In addition, we found that in cells probed for K48, while Parkin was found to be clustered perinuclearly, K48 chains appeared to be sequestered away from the nucleus, possibly signaling for proteasomal degradation in other parts of the cell after degradation of its OMM substrates. In contrast, K63 chains appeared to colocalize with Parkin perinuclearly throughout the same timeframe. While further experiments must be conducted to validate the responses of these chain types over time to continuous mitochondrial depolarization, our results suggest that phosphorylated K63 chains likely serve as lasting markers of mitochondrial damage.

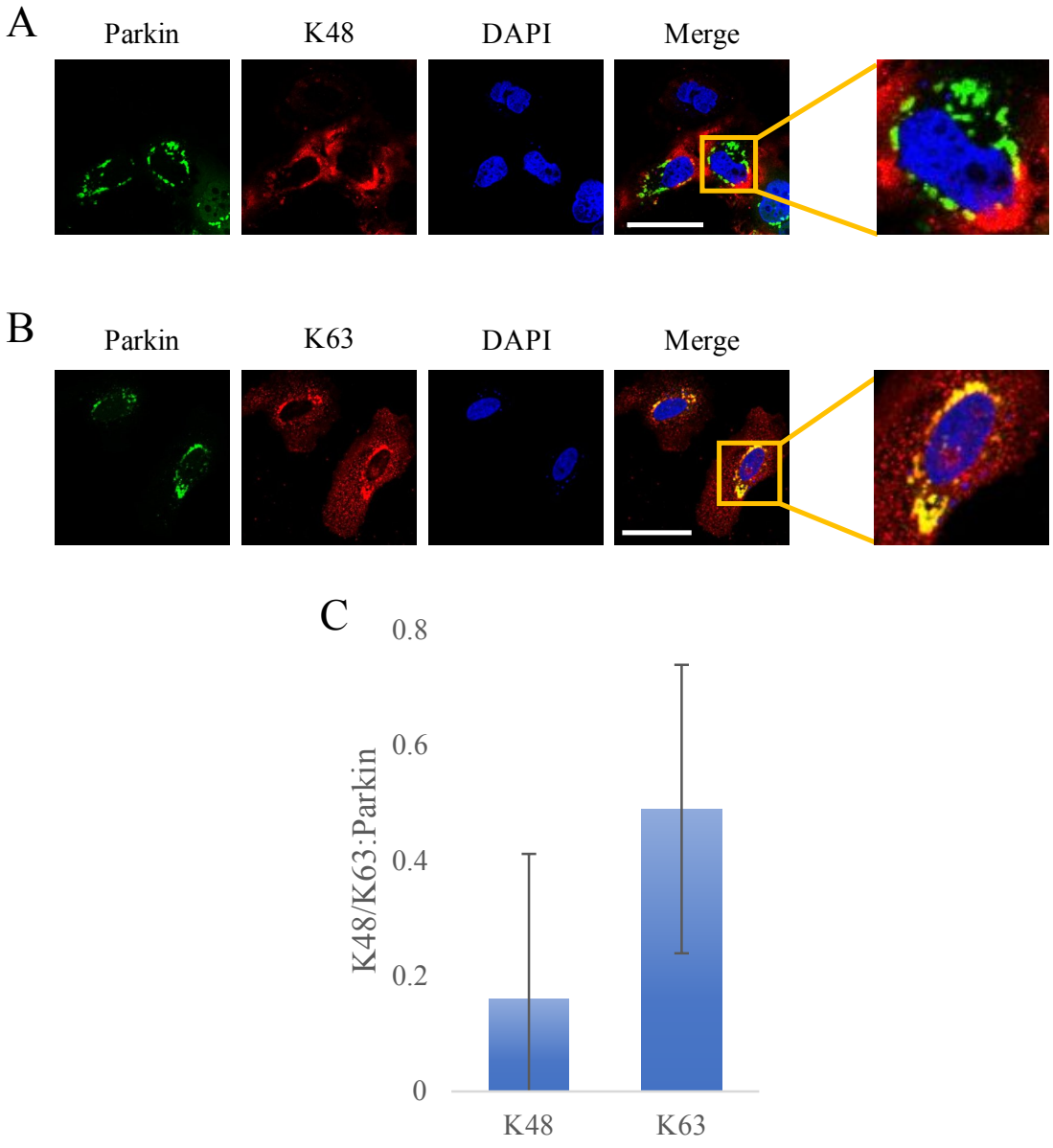


Figure 9: K48 pUb chains are less colocalized with Parkin than K63 chains after 3 hours of continuous 10 μM CCCP treatment. Fluorescence microscopy images of HeLa cells expressing EYFP-Parkin (green), fixed at 180 minutes during CCCP treatment and stained with NucBlue (blue) and either (A) K48 antibodies (red) to detect K48 polyubiquitin or (B) anti-K63 antibodies (red) to detect K63 polyubiquitin. Scale bars represent 50 μm. (C) The colocalization of EYFP-Parkin and K48 or K63 was assessed using Pearson's correlation coefficient (PCC) and graphed as the average PCC score. Data is from one biological repeat with a minimum of 32 cells per condition. Error is represented as S.D.

IV. DISCUSSION

The PINK1:Parkin pathway is a mitochondrial quality control system that responds to a range of mitochondrial insults, destroying only those mitochondria posing an imminent threat to the health and integrity of the cell's mitochondrial network and the viability of the cell itself. The responses of PINK1 and Parkin together provide the cell with real-time feedback on even minor decreases in MMP, initiating the destruction of persistently depolarized mitochondria (reviewed in Harper *et al.,* 2018). The interactions between PINK1, Parkin, and downstream autophagy regulators have been extensively scrutinized under persistent stress conditions in *in vitro* cell models in which the cell is subjected to complete loss of MMP for extended periods of time (Fiesel *et al.,* 2015, Heo *et al.,* 2015, Moore and Holzbaur, 2016, Padman *et al.,* 2019). Although this research has been invaluable in our understanding of the basic functioning of the pathway, little is known about how the pathway responds to physiologically relevant perturbations, including transient or partial loss of MMP, as would be expected to occur *in vivo*.

The model of the PINK1:Parkin pathway currently represented in the literature appears binary in nature – the pathway is either 'off' during unstressed conditions or 'on' under persistent levels of stress with regulatory factors recruited in a predictable, step-by-step fashion (Figure 1). If the mitochondria are sufficiently depolarized for long enough periods of time, the result is always mitochondrial degradation. While experiments conducted in this manner have provided information about the identity of proteins involved at each step, where they are localized, and the sequence in which they associate with mitochondria, this model fails to describe how the system might behave under more nuanced circumstances, such as partial depolarization of the mitochondria or spontaneous

fluctuations in MMP, as has been observed in many cell types, including cultured neurons (Buckman and Reynolds, 2001), or even MMP oscillations in stressed cardiomyocytes (Aon *et al.,* 2003).

The multiple interlocked network motifs that are contained within the PINK1:Parkin pathway (Figure 4) strongly suggest that the system will not behave in a linear fashion. The role of the two coherent feedforward loops in the system (Figure 4A, C) is to create a delay in the response, initially slowing the formation of difficult-to-remove ppUb chains at the OMM on loss of MMP. PINK1 and Parkin must both be recruited to the mitochondria in sufficient quantities to facilitate the efficient assembly of ppUb chains on OMM proteins, and even then, the presence of ppUb chains are not representative of a full commitment to mitophagy as they are not invulnerable to dephosphorylation and deubiquitination (Bingol *et al.,* 2014, Wang *et al*., 2018).

Previously published data shows the rapid accumulation of full-length PINK1 on the mitochondria, followed by complete loss of PINK1 upon MMP recovery (Matsuda *et al.,* 2010). However, studies have failed to address how the pathway might respond to partial loss and partial restoration of MMP. Single-cell PINK1 imaging data from the Nelson lab has shown that partial MMP recovery is enough to cause PINK1 dissociation from the mitochondria (unpublished data). Because these experiments were conducted using exogenous PINK1-EGFP constructs, it was unclear whether untagged, endogenous PINK1 would behave similarly. It was expected that the western blot experiments using endogenous PINK1 would produce similar results to the exogenous PINK1 imaging experiments, with partial recovery of MMP resulting in PINK1 dissociation from the mitochondria; however, the partial recovery dose of CCCP (5 μM) yielded a more

confounding response. Across 3 repeats of this experiment, the 5 μM CCCP recovery samples varied greatly, with our densitometry analysis showing a PINK1 concentration pattern more similar to the continuous 10 μM samples than our 2.5 and 0 μM recovery concentration samples (Figure 5B, C, Appendix A).

We also found that, as expected, PINK1 remains stabilized at the mitochondria under persistent total mitochondrial depolarization and is cleared within 30 minutes of mitochondrial repolarization with 0 and 2.5 μM CCCP (Figure 5B, C). Since the results for the 10-to-5 μM CCCP treatment varied so greatly, this suggests that there is some threshold for MMP, below which, PINK1 remains stable, but above this threshold, PINK1 is imported through the TIM transporter and degraded. We believe that the 5 μM CCCP treatment will bring MMP very close to this threshold in most cells, allowing individual cells to exhibit different behaviors within the same population. This threshold is likely a function of the TIM transporter, as it is highly sensitive to MMP (Malhotra, *et al.,* 2013). Therefore, we propose that PINK1 stabilization may be affected by reaching a certain threshold of MMP, and when cells are brought close to this critical threshold through incubation in 5 μM CCCP, PINK1 behavior likely becomes highly variable between cells. After reducing CCCP concentrations to 2.5 or 0 μM, MMP will exceed the threshold required for TIM function and, therefore, PINK1 is efficiently imported through the transporter and degraded. In light of this experiment's results, an obvious next step in future investigations would be to more precisely determine the threshold MMP value for PINK1 stabilization/clearance. This could be done through careful titration of CCCP, increasing or decreasing the concentration with smaller increments.

Because PINK1 can be completely removed upon repolarization, this suggests that the mitophagy pathway is able to reset after transient insults at the PINK1 level. We next chose to investigate the responses of PINK1 to repeated mitochondrial insults to investigate whether PINK1 response is influenced by prior events. We felt that this was an interesting question as some cell signaling pathways can become sensitized or desensitized to subsequent stimuli by prior exposure to the same stimulus. For example, a recent study by Adamson *et al*. (2016) showed that upon pulses of the cytokine, TNFα, the NF-κB signal transduction pathway experiences a refractory period, during which time consecutive pulses of the cytokine result in a decreased response of the downstream protein, IκBα. In agreement with live cell data (unpublished data from the Nelson lab), repeated pulses of CCCP did not appear to influence PINK1's rapid clearance from the mitochondria. Our data showed that PINK1 levels returned to the baseline within 40 minutes of recovery via CCCP wash-out (Figure 6B). Our data did, however, contradict live cell experiments by indicating a statistically significant higher concentration of PINK1 recruitment after subsequent pulses of CCCP (p value = 0.011; Figure 6C). We believe the discrepancy between the live cell imaging experiments and western blot data may be a consequence of the expression of exogenous PINK1 in the live cell experiments as well as differences in measuring PINK1 response between the two experiment types. Because the PINK1-EGFP construct used contains a cytomegalovirus (CMV) reporter, PINK1-EGFP is expressed constitutively throughout the course of the experiment; however, while we still expect cells expressing only endogenous levels of PINK1 to maintain a constant rate of PINK1 expression, these cells may transcriptionally upregulate PINK1 upon induction of the mitophagy pathway, making them better able to

respond to further MMP loss. The method in which 'peak' PINK1 levels were measured also differed between live cell imaging and western blot experiments. Live cell imaging techniques allowed for higher temporal resolution, enabling quantification of peak PINK1 levels in individual cells, whereas our western blot experiments only provided an average of PINK1 expression among cells at a fixed timepoint. However, between both the live cell and western blot experiments, we did not observe evidence of PINK1 undergoing a refractory period between CCCP pulses as is common in other systems (i.e. the NF-κB pathway). This shows that PINK1 simply serves as a reporter of current MMP, an important role in mitochondrial quality control.

Overall, the behavior of PINK1 accumulation and dissociation from the mitochondria upon repeated, transient mitochondrial insults affirms its role as a direct reporter of changes in MMP – when MMP is lost, PINK1 remains in the OMM until normal MMP is at least partially restored to levels that permit PINK1 import into the TIM transporter. This very sensitive quality control mechanism may be vital for the prevention of inappropriate mitophagy responses to small or short-lived changes in MMP. Collectively, the PINK1 pulsing experiments suggest that prior depolarization does not affect, or possibly slightly enhances, PINK1 stabilization on subsequent depolarization events; however similar live cell experiments conducted by the Nelson lab that examined Parkin instead of PINK1 demonstrated that while PINK1 responds quickly to mitochondrial repolarization, Parkin does not (unpublished data; Figure 10). During successive pulsing experiments, Parkin was much slower to dissociate from the mitochondria and accumulated there in a step-like manner, increasing to higher levels with each CCCP pulse. These results suggest an anchoring of Parkin, but not PINK1, to

the mitochondria after mitochondrial repolarization. Previous studies have suggested that active Parkin tethers itself to ppUb chains on the mitochondria (Ordureau *et al.,* 2014), possibly causing this accumulation of Parkin, along with ppUb, over time.

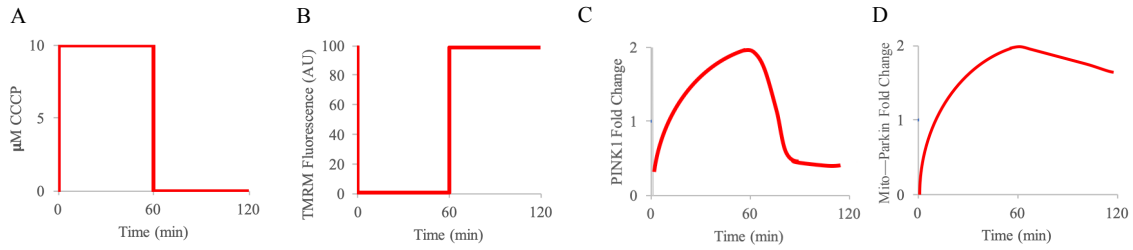


Figure 10: PINK1 is quick to dissociate from the mitochondria, while Parkin slowly dissociates. Live cell experiments show that when cells are treated with (A) the above concentrations of CCCP to (B) completely depolarize the mitochondria, (C) full-length PINK1 levels rapidly decline after media replacement, while (D) Parkin levels remain high even after CCCP wash-out. Redrawn data from Nelson Lab.

Studies suggesting that ppUb is more resistant to deubiquitinases than its precursor, pUb (Wauer, *et al.,* 2015), as well as our current knowledge of Parkin's association with ppUb (Ordureau *et al.,* 2014), led to our hypothesis that ppUb serves as a marker of prior mitochondrial stress and Parkin retention. Indeed, immunoblot experiments following the disassembly of total ppUb chains demonstrated that ppUb is removed from the mitochondria at a much slower rate than PINK1. In fact, whereas PINK1 was completely undetectable at 40 minutes post CCCP wash-out (Figure 6B), ppUb chains remained detectable by western blot 1-to-2 hours post CCCP wash-out (Figure 7). The observed delayed response of ppUb disassembly from the mitochondria, possibly due to the inefficiency of deubiquitinating enzymes to remove these chains (Bingol *et al.,* 2014), suggests that it may serve as a form of cellular 'memory,' enabling

the mitochondria to respond more quickly to subsequent damage. We anticipate that upon repeated pulses of CCCP, ppUb levels would continue to increase in a step-wise manner, increasing the likelihood of stable recruitment of autophagy receptors and the sequestration of the mitochondria into autophagosomes, which presumably constitutes a "point-of-no-return" and guaranteed mitophagy – a trend that was observed in Parkin upon repeated exposure to 30 minute CCCP pulses spaced by 30 minutes between pulses (unpublished data from the Nelson lab). When 30 minute CCCP pulses were spaced by 120 minutes, Parkin returned to basal levels between pulses, suggesting that Parkin is removed from the OMM and, more specifically, from ppUb chains. As it has been suggested that Parkin is tethered to ppUb on the OMM (Ordureau *et al.*, 2014), we would expect experiments analyzing ppUb response to repeated pulses to follow similar trends to the Parkin data mentioned above, accumulating to higher levels in a step-wise manner after successive insults, provided that these occur sufficiently close together.

Continuous CCCP exposure experiments revealed that ppUb levels remained elevated throughout the course of the experiment, up to six hours. This result suggests that long-term exposure to mitochondrial stressors leads to a maintenance of these chains despite the likely recruitment of deubiquitinases and phosphatases to the OMM (Bingol *et al*., 2014, Wang *et al.,* 2018) and, thus, efficient formation of the phagophore membrane around mitochondria. Should we extend the length of the experiment, we would expect to see sustained or increased concentrations of ppUb on the mitochondria until full phagophore engulfment of the damaged mitochondria. At this point, we imagine that mitochondrial ppUb levels may remain stable or at least would be unaffected by CCCP treatment or withdrawal, and will eventually be degraded upon lysosome fusion.

After obtaining our transient ppUb data, we next chose to pursue further investigation into two of the pUb chain types known to be conjugated by Parkin and the only ones which have been found to be further modified via phosphorylation by PINK1, K48 and K63 (Shiba-Fukushima *et al.,* 2014). Because these two chain types have different cellular functions, with K48 signaling for proteasomal degradation (Finley *et al.,* 1994) and K63 being involved in many processes, including DNA damage repair (Hofmann and Pickart, 1999, Pickart and Fushman, 2004), we expected the two chains to have varying roles and disassembly patterns in the mitophagy pathway as well. Disassembly of each of these chain types was examined using immunofluorescence to evaluate their individual responses to transient, 60-minute stress exposure with 10 μM CCCP by their colocalization with Parkin. These experiments revealed that while both K48 and K63 chains remain colocalized with Parkin at the mitochondria at least one hour after CCCP wash-out, K48:Parkin colocalization returns to basal levels just 2 hours post CCCP wash-out, while K63 colocalization with Parkin plateaus above the baseline 2 hours post CCCP wash-out, and remains elevated at least until 5 hours after repolarization (Figure 8). As K48 chains signal for proteasomal degradation, we speculate these chains are undergoing disassembly from the mitochondria more readily than K63 chains as proteasomal degradation occurs more rapidly than mitophagy, possibly as the K48 chains take their tagged substrate proteins to be degraded by the proteasome through the UPS, though the precise mechanism of phospho-K48 removal remains unclear. Proteins tagged by K48, such as mitofusins, are extracted from the outer mitochondrial membrane and transported to the proteasome through a process regulated by cytosolic AAA ATPases such as p97 in humans (reviewed in Karbowski and Youle,

2011). We believe that pK48 chains may, even at depolarized mitochondria, cycle between the phosphorylated and dephosphorylated states due to the presence of both PINK1 and PTEN-L. Each time a K48-polyubiquitinated chain is dephosphorylated provides an opportunity for its removal by both deubiquitinases and the proteasome; this is not the case for K63. In addition, K48 chains signal for proteasomal degradation in many other locations within the cell (Finley *et al.,* 1994); this sequestration of K48 chains elsewhere within the cell could influence the measured colocalization between these chains and Parkin – within the context of this assay, K48 presence in other parts of the cell essentially serves as 'background.' Because the function of K63 chains is to signal to autophagic receptors as ppUb, under continued mitochondrial depolarization events, we speculate that these chains remain tethered to mitochondrial membrane proteins until engulfment in autophagosomes occurs. We believe that K63 chains remain on the mitochondrial membrane as long as possible in order to complete their role of mitochondrial autophagy. In fact, upon initial evaluation of K48 and K63 under continuous total depolarization for 3 hours, K63 chains appear colocalized with Parkin at far greater levels after 3 hours of total MMP loss than K48 chains (Figure 9). Again, we anticipate this is possibly due to the removal of K48 during proteasomal degradation of their OMM substrate proteins, and the sustained tagging of K63 to its mitochondrial membrane proteins until mitophagy – a process that can take hours to complete (Bampton *et al*., 2005). Additionally, preliminary K48 colocalization data showed that K48 disassembly appears to occur at similar rates regardless of whether cells are transiently or continuously stressed with 10 μM CCCP, and K63 levels remain consistently above their baseline levels across experiments (Figures 8 and 9). While these data are preliminary,

and these results need to be reproduced, this new insight into the variation in kinetics between the two chain types is quite interesting; the continuous depolarization experiment suggests that while both K48 and K63 chains are possible targets for PINK1 phosphorylation based on prior *in vitro* studies (Okatsu *et al.,* 2015), K48 chains undergo disassembly at much quicker rates than K63 chains. This provides further evidence that phosphorylated K63 chains may be most responsible for Parkin retention in the case of MMP fluctuations or subsequent mitochondrial insults. If cells were exposed to a second mitochondrial depolarization event, we would expect K63 levels to build in a step-wise manner with each subsequent pulse, as we believe they serve as docking sites for autophagic adapters, and we would expect to see some K48 chain conjugation on the OMM, though in less abundance than after the first pulse of damage as, with a short amount of recovery time between pulses, most of its substrate proteins would have been degraded after the first mitochondrial insult. If more time for mitochondrial recovery is provided between insults, we might expect higher concentrations of K48 chain conjugation upon consecutive pulses of CCCP.

Our findings through this study have shown that the current view of the mitophagy pathway depicted in much of the available literature does not encompass the more complex behaviors described in our study, showing that the pathway can integrate information about MMP over time and possibly identify the mitochondria on the cusp of failure. In these cases, the slow accumulation of Parkin likely results in slow rates of mitophagy. In fact, our data show that while PINK1 may be stably recruited to the mitochondria under consistent, complete MMP loss, it is quickly cleared upon MMP recovery, regardless of prior mitochondrial insults. Our data also demonstrate that unlike

PINK1, ppUb removal from the mitochondria is quite slow, and while K48 levels return to the baseline over time, K63 remains colocalized with Parkin on the mitochondria at least 5 hours after CCCP wash-out. Together, these data provide further insight into the responses of the mitophagy pathway to MMP fluctuations and transient MMP loss, as might be expected as a normal part of cellular aging or damage. Specifically, these results point to a system that is able to distinguish between transient, short-lived changes in MMP while reserving the majority of the mitophagy machinery to cells experiencing continual, sustained mitochondrial damage, or those experiencing frequent, drastic fluctuations in MMP.

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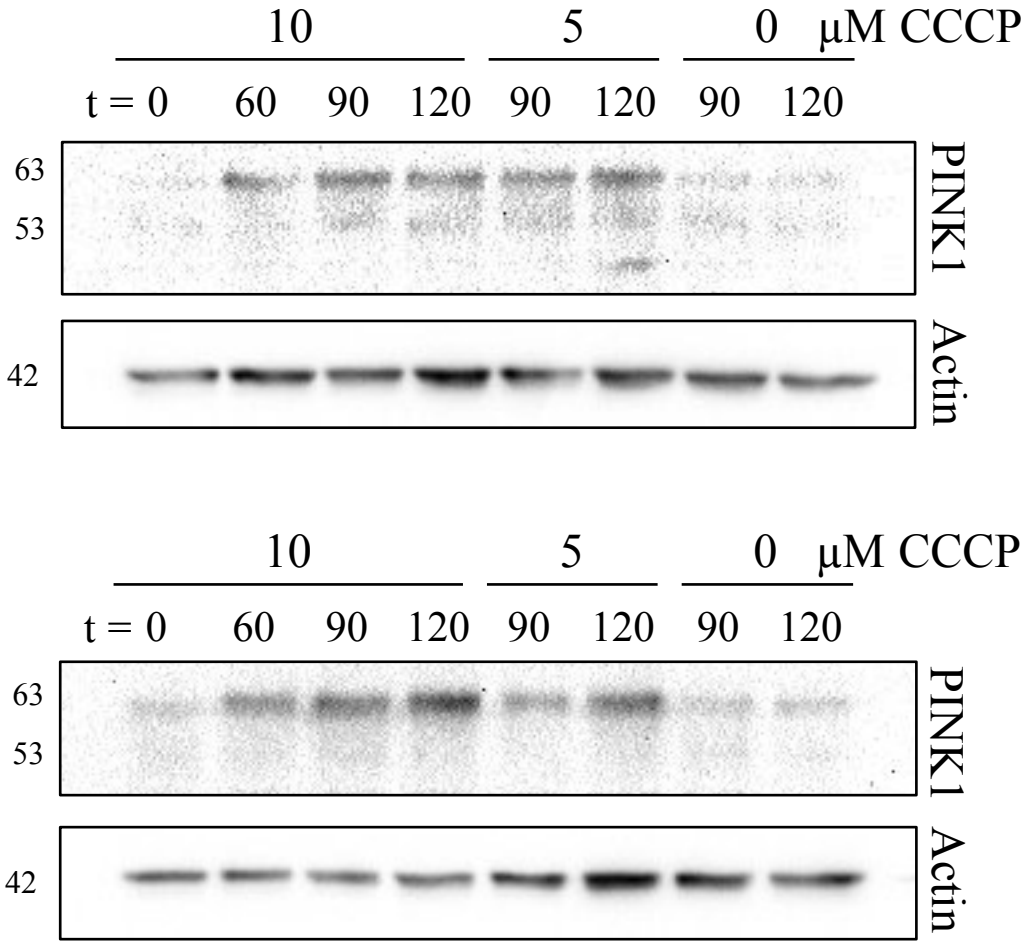
APPENDICES

APPENDIX A. Western blot repeats 2 and 3 from the experiment in Figure 5. HeLa cells were treated with the indicated doses of CCCP for the timescales provided. Immunoblots were probed with antibodies against endogenous PINK1 and actin for a loading control.



APPENDIX B. Full statistics from Figure 6C. PINK1 levels were quantified by densitometry using western blot data over 3 experiments was quantified using ImageJ software (NIH). Error bars represent S.E. Statistical significance is represented as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (one-way ANOVA and Tukey's post-hoc test), N = 3.