Use of a live cell microscopy approach to compare the response of the PINK1:Parkin mitophagy pathway to different mitochondrial depolarization agents

by

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ABSTRACT

The PINK1:Parkin pathway regulates mitochondrial network health by facilitating the autophagic destruction of damaged or dysfunctional mitochondria. While this pathway is widely studied, it remains unclear how it responds to recurring or partial losses of mitochondrial membrane potential. Here we show that PINK1 is capable of a rapid response to fluctuations in mitochondrial membrane potential. Parkin, using phospho-ubiquitin as a docking site, serves as a marker of previous mitochondrial stress and can accumulate over time so long as its ubiquitin ligase activity is conserved. The two different time scales on which these proteins operate ensure immediate responses to MMP fluctuations and incorporation of mitochondrial stress over time.

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ABBREVIATIONS

BAM15- N5,N6-bis(2-Fluorophenyl)[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine

CCCP- Carbonyl cyanide *m*-chlorophenyl hydrazone

DMEM- Dulbecco's Modified Eagle's Medium

EGFP- Enhanced Green Fluorescent Protein

Mfn1- Mitofusin 1

Mfn2 - Mitofusin 2

MMP-Mitochondrial Membrane Potential

MPP- Mitochondrial Processing Peptidase

MTS-Mitochondrial Targeting Sequence

NDP52- Nuclear Dot Protein 52

OMM- Outer Mitochondrial Membrane

OPTN- Optineurin

PARL- Presenilin-Associated Rhomboid-like Protein

PD- Parkinson's Disease

PEI- Polyethylenimine

PINK1- PTEN induced putative kinase 1

ppUb- Phospho-polyubiquitin

TIM- Translocase of the Inner Membrane

TMRM- Tetramethylrhodamine, Methyl Ester, Perchlorate

ROI- Region of Interest

ROS- Reactive Oxygen Species

TOM- Translocase of the Outer Membrane

VDAC- Voltage Dependent Anion Channel

YFP- Yellow Fluorescent Protein

I. INTRODUCTION

1.1 The Importance of Mitochondrial Quality Control

The maintenance of healthy mitochondrial networks is essential for a variety of fundamental cellular metabolic processes. As such, mitochondrial networks must routinely expunge dysfunctional regions to prevent the propagation of damage across the network, and to avoid affecting other cellular processes or the integrity of the cell as a whole. While there are numerous molecular mechanisms that operate collectively to maintain mitochondrial networks (e.g. biogenesis, fission-fusion, mitochondrial unfolded protein response), the PINK1:Parkin pathway regulates what could be the most extreme form of mitochondrial quality control: the autophagic destruction of damaged, depolarized mitochondria.

The PINK1:Parkin pathway functions by responding to losses in mitochondrial membrane potential (MMP or $\Delta\Psi m$), a key indicator of mitochondrial stress, and is notable for its association with heritable forms of Parkinson's Disease (PD). In the absence of a fully functioning PINK1:Parkin pathway, as seen in PD patients with autosomal recessive mutations in genes encoding Parkin (*PARK2/PRKN*) and PTEN induced putative kinase 1 (PINK1) (*PARK6/PINK1*), a significant loss of dopaminergic neurons within the substantia nigra of the midbrain is observed. As these neurons have unusually large axonal arborizations and likely place high metabolic demands on their mitochondria, it seems likely that the PINK1:Parkin-directed mitochondrial quality control is essential for the health of these cells (Pickrell & Youle, 2004).

Mitochondria are regularly exposed to stressors that are capable of interfering with the native state of the mitochondrial membrane, such as exposure to reactive oxygen species (ROS), which are generated as a byproduct of normal metabolic processes (Vergun & Reynolds, 2004). These stresses may affect the ability of mitochondria to maintain a proton gradient across the inner mitochondrial membrane (IMM) and may therefore induce a partial or complete loss of mitochondrial membrane potential (MMP or $\Delta\Psi m$). As damage to discrete mitochondria can potentially propagate throughout a cell's mitochondrial network or promote cell death through the uncontrolled release of apoptotic regulators, it is vital that cells have a mechanism to rapidly identify, isolate, and destroy irretrievably damaged mitochondria (Wang & Youle, 2009).

1.2 Quality Control Mechanisms for Damaged Mitochondria

Changes in or a loss of MMP is detected by PINK1, which is a highly labile nuclear-encoded serine kinase. In healthy mitochondria, the N-terminus of PINK1 is imported into the mitochondrial matrix through the combined action of translocases of the outer membrane (TOM) and the $\Delta\Psi m$-dependent translocases of the inner membrane (TIM). Here, the N-terminal mitochondrial targeting sequence (MTS) is removed by mitochondrial processing peptidase (MPP) and PINK1 is cleaved within the transmembrane domain (TMD) by presenilin-associated rhomboid-like protein (PARL) (Greene et al., 2012). The now 52 kDa protein is ubiquitinated at the outer mitochondrial membrane (OMM) and targeted for destruction by the 26s proteasomes (Liu et al., 2017). This process of routine cleavage keeps PINK1 at a low-level state within the cytosol of

cells with a healthy mitochondrial network (Pickrell and Youle, 2015). However, at damaged mitochondria that have suffered a loss of MMP, TIM-dependent import of PINK1 is blocked, preventing cleavage of the protein by MPP and PARL (Jin et al., 2010). This results in the accumulation of full-length PINK1 proteins at the OMM of depolarized mitochondria (Jin and Youle, 2012), forming large TOM-associated complexes with the C-terminal kinase domain of PINK1 facing outwards into the cytosol (Yamano et al., 2016).

PINK1 accumulation on the surface of the outer mitochondrial membrane coincides with an increase in PINK1 kinase activity, where it phosphorylates cytosolic Parkin proteins at Ser65 within a regulatory N-terminal ubiquitin-like domain (Ubl) as well as phosphorylating ubiquitin moieties conjugated to OMM proteins, also at Ser65. The phosphorylated ubiquitin essentially serves as a receptor for Parkin, enabling it to dock and accumulate on the cytosolic face of the OMM.

The phosphorylation of Parkin at Ser65 in its RING1 domain has been proposed as a mechanism for releasing Parkin from an autoinhibitory state. Upon release of this autoinhibitory state, catalytic cysteine residues are exposed following a conformational change, resulting in Parkin activation. Phospho-ubiquitin molecules and phosphorylation of the protein itself stimulate Parkin E3 ubiquitin ligase activity with optimal activation requiring both, as demonstrated in *in vitro* studies using S65A Parkin and ubiquitin mutant proteins (Kazlauskaite et al., 2014; Kane et al., 2014).

Activated Parkin decorates the OMM with polyubiquitin chains and is conjugated to a broad range of protein substrates, including mitofusin 1 (Mfn1), mitofusin 2 (Mfn2),

and voltage dependent anion channel (VDAC) amongst other mitochondrial substrates (Scarffe et al., 2014). Ubiquitination of these mitochondrial substrates act as a targeting mechanism of proteasomal degradation and, as a consequence, alter the fusion and fission dynamics of the mitochondrial network. This change in dynamics has been implicated in the resulting perinuclear clustering seen following sustained depolarization of the mitochondrial membrane. As the rate of fission begins to outweigh the rate of fusion, the smaller mitochondrial fragments that result from this imbalance are much easier to transport than larger pieces (Itoh et al., 2013).

The ubiquitin subunits of the poly-ubiquitin chains formed by Parkin are also phosphorylated by PINK1, which allows for further activation and recruitment of Parkin. This creates a positive feedback loop whereby Parkin and poly-ubiquitin chains are increasingly recruited and assembled at depolarized mitochondria (Durcan and Fon, 2015), resulting in a rapid formation of a phospho-polyubiquitin (ppUb) coat at the OMM of depolarized mitochondria, which appears to become saturating within ~60 min post-depolarization. However, this process is essentially reversible if interrupted at its earliest stages through the activities of de-ubiquitinase enzymes such as Ubiquitin carboxyl-terminal hydrolase 30 (USP30) and other mitochondrial USPs (Bingol et al., 2014). These enzymes, as the name suggests, function to remove ubiquitin from the outer surface of the mitochondrial membrane. However, it has been noted that these enzymes are far less effective on ubiquitin that has been phosphorylated, rendering these enzymes ineffective over long periods of sustained mitochondrial membrane depolarization. In more recent studies, it has also been found that ubiquitin phosphatases such as PTEN-L

slow down the progression of mitophagy by dephosphorylating p-Ser65, thereby reducing Parkin docking sites (Wang et al., 2018).

The building of these ubiquitin networks at the surface of the mitochondrial membrane also facilitates the recruitment of autophagy adaptors to the mitochondrial membrane. PINK1 has been shown to be essential for the recruitment of both optineurin (OPTN) and nuclear dot protein 52 (NDP52) to the OMM. If there is a lack of kinase activity by way of PINK1, neither OPTN nor NDP52 are recruited to the OMM, as can be seen in systems where PINK1 has been replaced with kinase dead PINK1 (PINK1-KD) (Lazarou et al., 2015). OPTN recruitment is followed by the recruitment and activation of tank-binding kinase-1 (TBK-1), which, in turn, phosphorylates the ubiquitin and LC3 binding domains of both p62 and OPTN and also phosphorylates sites on NDP52 and TAX1BP1.

As these ubiquitin networks are built on the outer mitochondrial membrane in tandem with the recruitment of autophagy adaptors, the autophagosome is formed around the mitochondria. The LC3 binding domains of these recruited components can now be utilized as docking regions for the autophagosome, which is eventually followed by fusion with lysosomes, resulting in the degradation of the mitochondrial fragment within.

Although the various components of the mitochondria have been well characterized, as have the various pathogenic PINK1 and Parkin mutations associated with autosomal recessive forms of Parkinson's Disease, questions remain about how the pathway responds to different types of mitochondrial insult. Despite having a fair understanding as to how these proteins operate and respond to mitochondrial

perturbances, it is still largely unknown how the mitophagy pathway integrates more complex inputs. The activity of the PINK1: Parkin pathway and the dynamics of its protein components are typically studied only under the conditions of total loss MMP (Park et al., 2014; Kondapalli et al., 2012; Vives-Bauza et al., 2010; Narendra et al., 2010). It is therefore unclear as to how the pathway may respond to a partial loss of or fluctuations in MMP, or whether prior mitochondrial insults can affect the response of the pathway to subsequent stresses. These constitute important questions as mitochondrial activity has been shown to slowly decline over time as a natural part of the aging process. Furthermore, various cell types exhibit periodic changes in MMP as part of basic cellular functions or in response to stress.

1.3 Study Aims

The current understanding of how the PINK1:Parkin pathway operates is derived primarily from experiments that subject the system to continual stress, with a large focus on how the pathway proceeds as one protein recruits the next. While this is effective in building a clear picture of how this pathway proceeds in a more linear sequence of events, it fails to show how this pathway responds to more complex signals. The types of mitochondrial stresses produced *in vivo* will be time-varying (i.e. transient, sustained, or repeating) and will differ in magnitude and nature (i.e. oxidative damage and/or depolarization). Taking this into consideration, a more simplistic method of observing this pathway under sustained stress may not be the most effective if we are to fully understand the properties of the pathway. As such, most of our experiments are designed in such a manner to see how this pathway acts under conditions that deviate from the

most common approaches that are used by many labs. It is currently unclear how the mitophagy pathway will respond to a sustained partial loss of MMP. Furthermore, the current literature fails to address how this pathway responds to recurrent complete losses of MMP.

To assess how the mitophagy pathway responds to recurring bouts of complete loss of MMP, we exposed the cells to transient pulses of 10μM Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at varying intervals while simultaneously measuring the levels of fluorescently tagged PINK1 and Parkin proteins at mitochondria using fluorescence microscopy. We hypothesized that, since Parkin utilizes ppUb, which is relatively long-lasting and resistant to degradation, as a docking site, Parkin should be maintained at the mitochondrial membrane for periods long after the initial depolarization of the mitochondrial membrane. As PINK1 does not stably associate with ppUb and is retained by different mechanisms, we also hypothesized that it would dissociate from the OMM and be destroyed even after partial or transient recovery of MMP.

As a method to test our hypothesis, we also produced plasmid constructs to express the fluorescently tagged R275W Parkin mutant. While this mutant is essentially E3-ligase dead, like the majority of PD-associated Parkin mutants, it is unique in that it is recruited to the OMM with near identical kinetics to WT Parkin proteins. This tool would therefore enable us to assess the importance of ppUb for Parkin retention after repolarization of mitochondria. Finally, as a method of validating our results, we repeated some key experiments with, N5,N6-bis(2-Fluorophenyl)[1,2,5]oxadiazolo[3,4-

b]pyrazine-5,6-diamine (BAM15) which is a mitochondrial uncoupler with comparable effects to CCCP.

II. MATERIALS AND METHODS

2.1 Cell Culture

HeLa (CCL-2) cells, an immortal cervical carcinoma cell line, were obtained from ATCC (USA) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin solution and maintained in a humidified incubator at 37°C under 5% CO_2 concentration. These cells were selected for this study as they are effectively Parkin-null and do not express detectable levels of the protein. For this reason, they are frequently used in the PINK1:Parkin field, enabling investigators to reconstitute the cells through transient transfection of plasmid constructs to express WT or mutant Parkin proteins.

2.2 Transfection

Cells were transfected with plasmid DNA using Polyethylenimine (PEI), a lipid-based reagent that complexes with plasmid DNA, enabling it to pass through the plasma membrane and enter the cell. PEI is stored at 4°C prior to use. Transfection mixes were prepared using a fixed ratio of 100 µL of unaltered DMEM combined with 3 µL of PEI reagent per microgram of plasmid. For experiments using fluorescently tagged PINK1, which were performed using glass-bottom 35 mm dishes (Cellvis, USA) containing approximately $1x10^5$ HeLa cells, 1.0 µg of the pPINK1-EGFP plasmid was co-transfected with 0.5 µg of plasmid mito-mCherry, which encodes an mitochondria-targeted version of the red fluorescent protein, mCherry. PINK1 experiments were also performed using 0.5 µg of pPINK1-mCherry plasmid co-transfected with 0.5µg pEGFP-

C1, encoding untagged, free EGFP. For Parkin experiments, HeLa cells were co-transfected with 0.5 μg of mito-mCherry with 0.5 μg of pYFP-Parkin. Transfection reagents were washed off after 6 hours and the cells were allowed to recover for a period of 24 hours before further manipulation and imaging.

2.3 Live Fluorescence Microscopy

For live cell microscopy experiments, HeLa cells were plated at 1×10^5 cells onto 35 mm glass bottom cell culture dishes in 2 mL of DMEM. Images were collected using a Nikon Ti-Eclipse wide-field fluorescence microscope, equipped with a CoolSNAP Myo camera (Photometrics, USA), Intensilight Epi-fluorescence illuminator, computer-controlled stage, and a fully enclosed incubation chamber with CO_2, humidity, and temperature control (InVivo Scientific, USA). Microscope control and image capture was facilitated using Nikon Elements Advanced Research Software (Nikon, USA) with images typically recorded at three-minute intervals. Pulsing experiments were performed by adding the mitochondrial membrane-depolarizing reagent CCCP to the media on the dish (10 μM) and subsequently removing and replacing the growth medium with fresh medium free from CCCP. Cells were washed twice with fresh media each wash before resuming imaging experiments. A minimum of three biologically independent repeats were performed for each experiment.

2.4 Image Analysis

For experiments involving PINK1-EGFP, individual cells were analyzed using Fiji (Schindelin et al., 2012). For PINK1 experiments, average EGFP fluorescence was measured within the bounds of a region of interest (ROI) that encapsulated the entirety of the cell. A background measurement was taken by measuring the fluorescence intensity in a region of the field that contains no cells, or cellular debris of equal area to the ROI. This background fluorescence measurement was subtracted from the ROI measurement. Parkin experiments were also analyzed using FIJI. In this case, a ROI measurement was taken at the site of a representative region of each cell's mitochondrial network, and a background measurement was also subtracted from these measurements. All data sets were normalized to T=0 minutes.

2.5 Two-Step PCR Mutagenesis and Amplification

To construct the pEYFP-Parkin-R275W expression plasmid, four different primers were utilized in a 2-piece mutagenesis strategy to introduce the missense mutation into the WT Parkin coding sequence (CDS). The forward and reverse primer for the Parkin CDS had *Bam*HI cut sites added to the 5' ends to facilitate cloning of the resultant PCR product into the multiple cloning site of the pEYFP-C1 plasmid (Takara/Clontech, USA). The two other primers utilized had the point mutation incorporated and were designed to anneal on the desired mutation location with 12 base pairs on either side of the mutation site. Two PCRs were run concurrently, both using the pEYFP-Parkin plasmid as a template, which contained the full-length WT human Parkin

CDS, with one utilizing the forward Parkin primer (5'GCATGGATCCATGATAGTGTTTGTCAGG 3') with the reverse mutation site primer (5' CGTGAACAAACTGCCAATCATTGAGTCTTGT 3'). The other contained the reverse Wildtype Parkin primer with a *Bam*HI cut site (5' GCAATGGATCCGCCACGTCGAACCAGTGGTCC 3') with the forward mutation site primer (5' ACAAGACTCAATGATTGGCAGTTTGTTCACG 3'). The two fragments retrieved following these reactions were combined and a PCR was run for ten cycles allowing these two fragments to anneal off of each other to create a full Parkin sequence with the mutation incorporated. This PCR fragment was amplified in the presence of the terminal forward and reverse primers for 30 cycles and then gel purified. A TOPO PCR cloning kit (ThermoFisher, Life Technologies, USA) was utilized to incorporate the PCR product into a shuttling vector. The resultant construct was amplified utilizing an EZNA Plasmid DNA Midi Kit (Omega Biotek, USA) and was subjected to restriction digest utilizing *Bam*HI to liberate the fragment encoding Parkin-R275W. This was separate from the plasmid backbone by DNA gel electrophoresis ligated into linearized, *Bam*HI-digested pEYFP-C1 plasmid using T4 DNA ligase. The ligation products were transformed into subcloning-competent DH5α *E.coli* and were amplified and purified via midi prep. The final product was validated via a diagnostic restriction digest using *Xho*I to verify proper assembly of the plasmid and orientation of the Parkin-R275W insert. This newly created plasmid was sequenced to verify there were no erroneous mutations introduced.

2.6 Statistical Analysis

One-way ANOVAs were utilized to compare data sets at selected time points. All experiments were performed in triplicate.

RESULTS

3.1 PINK1 is Capable of Rapid Dissociation from the Mitochondrial Membrane

Previous experimentation in our lab showed that when HeLa cells are exposed to 10 µM CCCP, a concentration that has been shown to cause a complete loss of MMP, PINK1-EGFP rapidly associates with the OMM, becoming saturated within an hour. When CCCP is removed from the cell growth medium, PINK1 dissociates from the mitochondrial membrane and is degraded within ~30 min, returning to basal levels (Bowling et al., 2019).

As one of the overarching aims of our lab is to determine how the PINK1:Parkin pathway interprets and responds to complex inputs, we decided to test whether the response of the pathway to depolarization at the PINK1 level was influenced by prior mitochondrial stresses. Given that PINK1 is rapidly degraded on recovery of MMP to near-undetectable levels and the cellular pool of PINK1 is effectively being constantly renewed and turned over, we hypothesized that the system would exhibit no 'memory' at the PINK1 level and the responses to separate insults would be fully independent, provided that PINK1 returned to basal levels prior to the next loss of MMP.

To test this hypothesis, cells that were transiently transfected with pPINK1-EGFP were exposed to 10 µM CCCP for 30 min followed by a recovery period with no CCCP for 30 min. This was immediately followed by a second 30 min 10 µM CCCP pulse. As expected, PINK1 levels in the cell increased in response to CCCP treatment. Upon removal of the CCCP, PINK1 levels returned to a low-level state within the cell. The CCCP

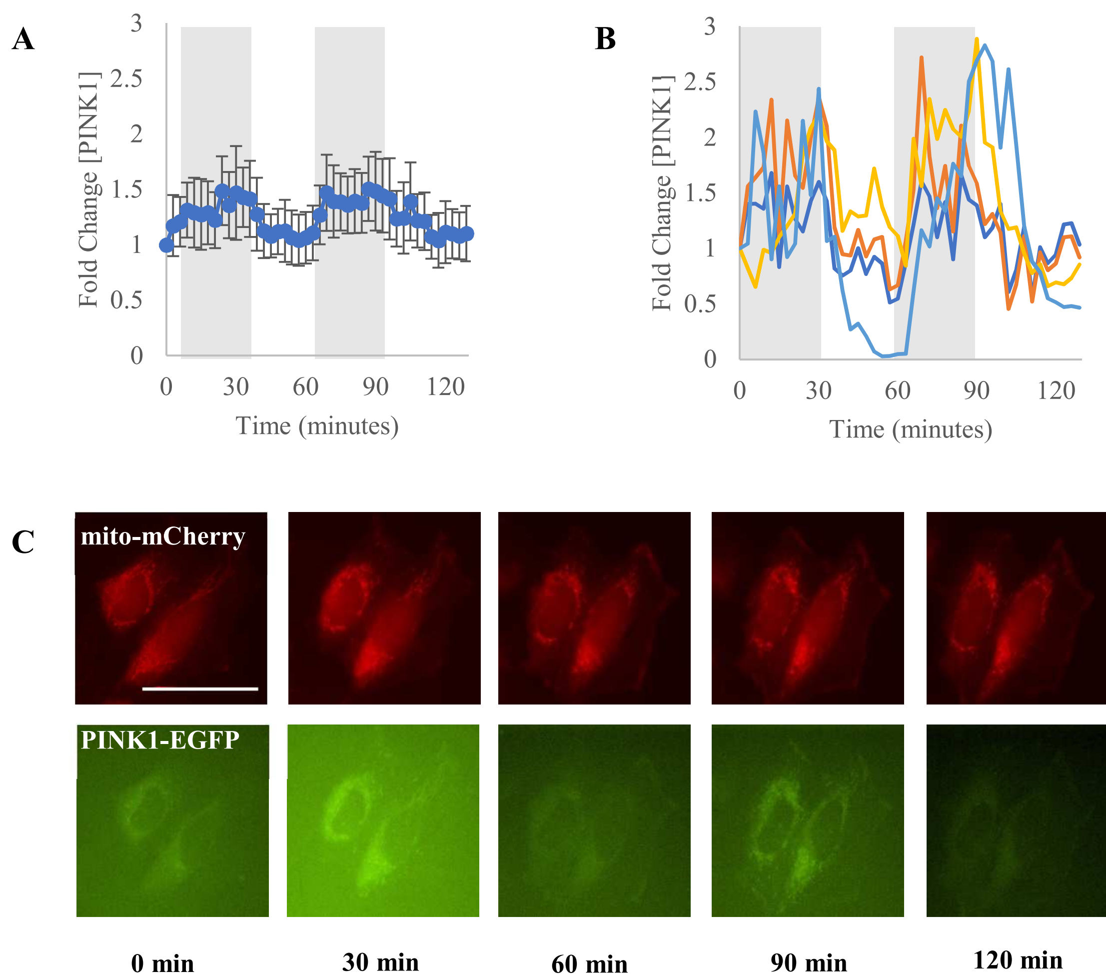

Figure 1. PINK1 levels respond quickly to changes in MMP PINK1 levels increase upon exposure to CCCP, but return to a low level state following the removal of CCCP (A). This pattern of behavior can be seen in both the single cell data (B) and the live fluorescence images (C). Gray areas represent 10μM CCCP exposure, while white area represents 0μM CCCP. Statistical analysis of PINK1 levels following recovery revealed no statistical significance when compared to time 0 levels ($p < 0.05$). Scale bar represent 50 μm.

dose that followed the first recovery period elicited a comparable PINK1 response to the first dosage of CCCP, followed by a similar decline in PINK1 levels upon CCCP washout (Fig. 1A-C). Statistical analysis revealed that the initial PINK1 response was not significantly different than that of the second response ($p < 0.05$). Furthermore, PINK1 levels following recovery from CCCP exposure were not statistically significant when compared to PINK1 levels at time zero ($p < 0.05$).

3.2 PINK1 exhibits distinct behaviors in response to partial and complete loss of MMP

Despite establishing the idea that PINK1 responds relatively quickly to increases or decreases in MMP, how this protein responds to different levels of persistent stress remains largely unclear. Previous data show that PINK1 will respond to a partial loss of mitochondrial membrane potential, albeit the response is transient and PINK1 levels decline without the removal of CCCP from the cells (Bowling et al., 2019). This contrasts the PINK1 response to a complete loss of MMP, which is marked by a sustained PINK1 response over the duration of the CCCP exposure. However, this type of behavior was observed on the relatively small time scale of two hours. To determine if such behaviors are consistent over longer time scales, PINK1-EGFP levels were measured in single cells over a 24-hour period in the presence of high or low concentrations of CCCP. As expected, PINK1-EGFP levels remained unchanged in cells that were not exposed to CCCP over the duration of the experiment (Fig. 2A+B). However, the cells exposed to 5 μM (Fig. 2A+C) and 10 μM CCCP (Fig. 2A+D) showed a very similar rate of PINK1 increase over the first 300 minutes of the experiment. While PINK1 levels continued to

increase in cells exposed to 10 µM CCCP, cells exposed to 5µM CCCP began to slowly decline for the remainder of the experiment. Cells exposed to 10 µM CCCP showed a slight decrease in PINK1 levels after a period of approximately twelve hours but remained significantly higher than basal levels for the remainder of the experiment. Furthermore, the slight upward trend in PINK1 levels towards the end of the experiment suggests that PINK1 levels could have been increasing again.

Despite the capacity of PINK1 to rapidly associate and dissociate from the outer mitochondrial membrane, previous experiments in our lab have shown that Parkin behaves in a distinctly different manner than PINK1 as it is retained at the surface of the outer mitochondrial membrane for periods much longer than PINK1 (Bowling et al., 2019). The disparity between the behaviors of these two proteins required that both PINK1 and Parkin be subjected to a variety of pulsing regimes to understand the differences in kinetics. While the dynamics of a PINK1 response have shown to be relatively simple in terms of how it responds to a loss of MMP, the slow rate of dissociation of Parkin makes its behavior harder to predict.

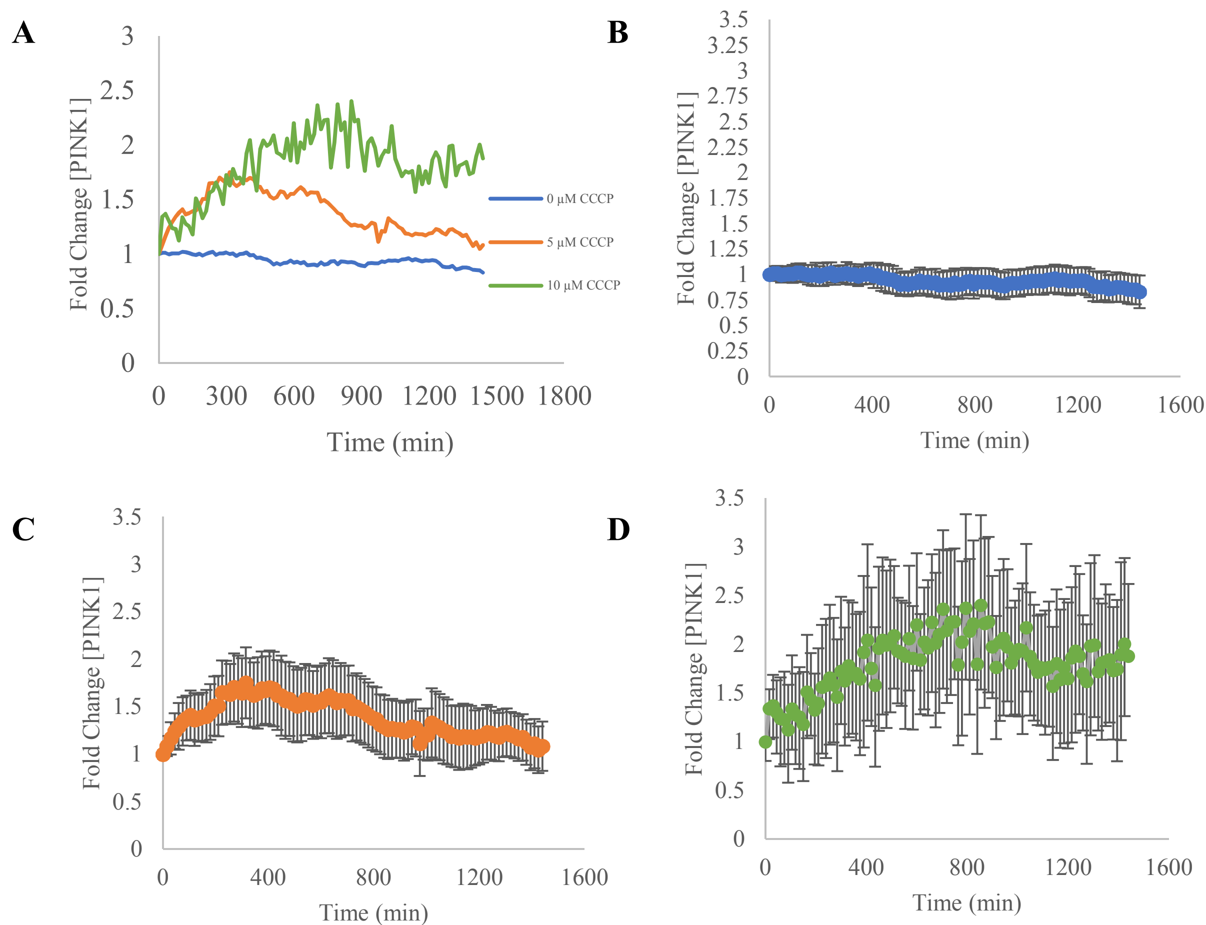

Figure 2. PINK1 exhibits a dose-dependent response to continuous CCCP treatment Controls cells that were not exposed to CCCP (0 µM CCCP) did not show notable changes in PINK1-EGFP levels over the 24 h imaging period (A+B). PINK1-EGFP levels increase upon exposure to both 5 µM CCCP (A+C) and 10 µM CCCP (A+D), but return to a low level state with continuous exposure to 5 µM CCCP. Statistical analysis of PINK1 levels comparing time zero to the final time point showed that PINK1 levels were significantly higher in cells exposed to 10µM CCCP but not in cells exposed to 5 or 0 µM CCCP ($p < 0.05$).

3.3 Parkin Accumulates in Response to Repeated, High Frequency Stress

Previous experimentation utilizing YFP-Parkin have shown that Parkin is retained at the surface of the outer mitochondrial membrane long after CCCP has been removed, remaining above basal levels for almost 2 h (Bowling et al., 2019). This suggested that, unlike PINK1, the response of Parkin to mitochondrial depolarization could be affected by earlier mitochondrial insults that occurred many minutes earlier and its concentration at the OMM would likely build to higher levels after each CCCP pulse.

To test this hypothesis, the same high frequency CCCP pulsing experiment depicted in figure 1 was repeated using HeLa cells expressing YFP-Parkin. As before, cells were incubated with 10 μM CCCP for 30 min followed by a recovery period two times consecutively. Unlike PINK1, Parkin levels do not return to a low-level state within the cell following the first exposure to CCCP. The second dosage of CCCP causes Parkin levels at the mitochondria to increase to levels significantly higher than that of the initial Parkin response, displaying a sort of 'building' effect (Fig. 3 A+B).

After showing the capacity of this protein to accumulate and build over time when periods of depolarization were spaced by relatively short periods of repolarization, we reasoned that this effect might be lost if the periods of repolarization were extended so that they were sufficiently long to allow OMM Parkin to return to prep-CCCP exposure levels. To test this, the experiment was repeated, extending the recovery period between CCCP pulses to 2 h, pulsing for a total of three times. Unlike the high frequency pulsing regime, this low frequency pulsing regime resulted in a return of Parkin to basal levels in the cell at the end of each recovery period (Fig. 3A+B).

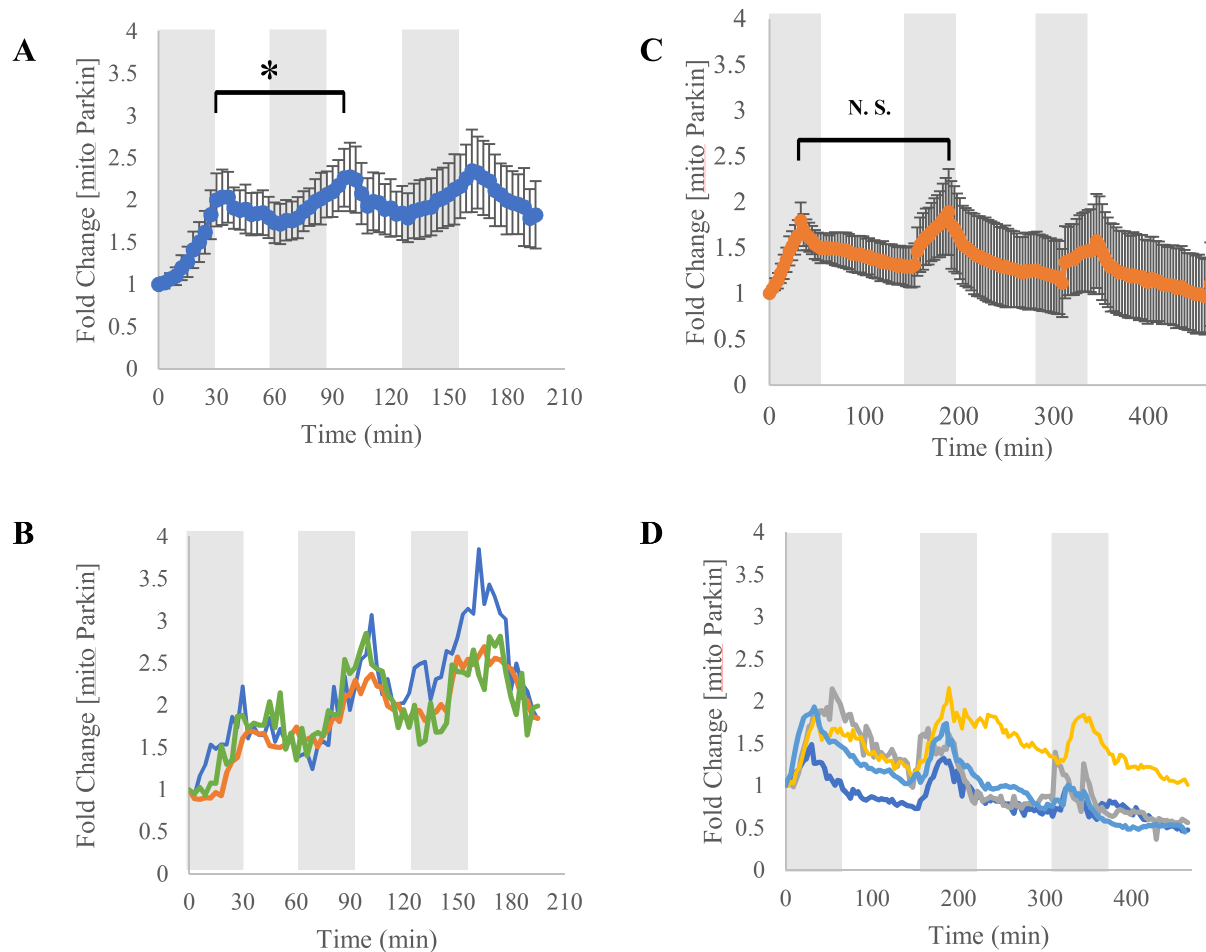

Figure 3. Parkin retention is dependent upon the frequency of exposure to CCCP Parkin levels are capable of returning to a low level state in the cell following a long recovery period, but increases when subjected to more frequent pulses of 10 µM CCCP. Thirty-minute exposure times coupled with thirty-minute recovery times resulted in increasing parkin levels (A), a behavior that can be teased out in the single cell data as well (B). However, thirty minute exposure times coupled with 120 minute recovery times resulted in parkin dissociation from the mitochondria (C & D). Gray areas represent 10 µM CCCP exposure, while white area represents 0 µM CCCP. Statistical analysis of parkin levels following recovery revealed a significant difference, but not for the low frequency pulsing experiment ($p < 0.05$).

Each Parkin response was comparable to the last, not increasing with each mitochondrial perturbance, but also not responding in a diminished capacity.

3.4 The R275W Mutation Alters Retention of Parkin at the Mitochondrial Surface

Amongst the many PD-associated mutations of Parkin that have been characterized, the R275W mutant is almost unique in that, while it exhibits a near-complete loss of E3 ubiquitin ligase activity, rendering the protein incapable of conjugating ubiquitin to the surface substrates of the mitochondria, it retains its ability to localize to the surface of the mitochondria. Furthermore, it is recruited to the OMM after mitochondrial depolarization with near identical kinetics to the WT protein. If the contrasting behaviors of PINK1 and Parkin in response to recurrent rounds of mitochondrial depolarization are associated with the docking of Parkin to ppUb chains at the OMM, it would be expected that the R275W mutant would be more rapidly lost from mitochondria after repolarization than WT Parkin.

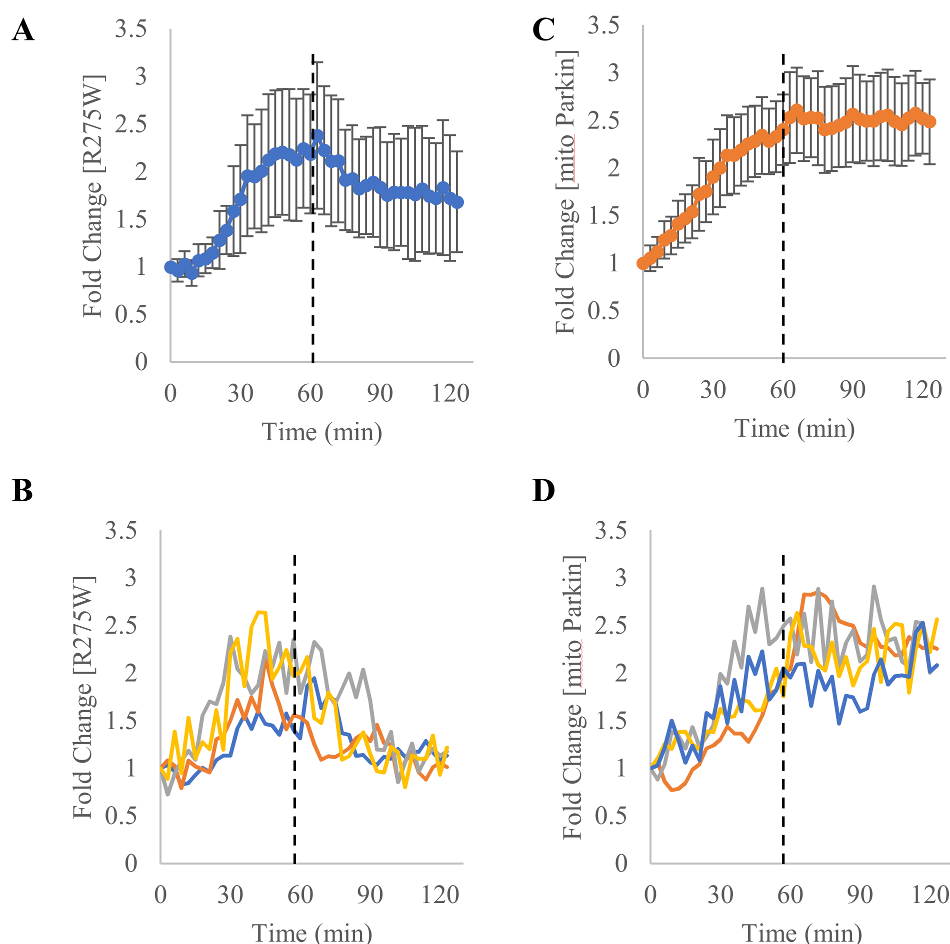

Figure 4. YFP-parkin and the R275W show different levels of retainment following CCCP washout Hela cells transfected with YFP-parkin or the R275W construct were exposed to 60 minutes of CCCP followed by a 60 minute recovery period. 30 minute exposures to these uncouplers, represented as gray areas, were followed by thirty minute recovery periods. The R275W mutant (A) does not increase in levels following multiple bouts of CCCP exposure whereas YFP-parkin (B) does. These two types of behavior are more evident in the single cell data, which shows a return in R275W to base levels (C) and building of YFP-parkin levels with each exposure (D). The difference in the two data sets is clear when plotted on the same graph (E). A peak analysis revealed a significant difference when comparing the second peak as a percentage of the first peak ($p < 0.05$). Data is from three biological repeats with a minimum of 30 cells per condition. Error is represented as S.E.M.

To test this hypothesis, the initial approach was to perform a simple one hour pulse with 10 μM CCCP followed by a one hour recovery period. Despite not being significantly different, there is an apparent difference in the way that the R275W mutant behaves when compared to YFP-Parkin. Upon removal of CCCP, YFP-Parkin shows no signs of decreasing over the following 1 h period of observation (Fig. 4C), while the R275W mutant begins declining (Fig. 4A). While the two distinct differences in behavior may not be as apparent in the averaged data, the single cell data displays rather clearly how R275W behavior is different from YFP-Parkin (Fig. 4B+D). While some cells transfected with the R275W mutant showed behavior more akin to YFP-Parkin, there was a tendency for Parkin-R275W levels to decline relatively quickly following CCCP washout.

The next approach was to see how the R275W mutant responds to a multitude of consecutive pulses as was previously tested with both PINK1-EGFP and YFP-Parkin (Fig. 1+3). While YFP-Parkin has the tendency to 'build' in levels with consecutive pulses of CCCP (Fig. 5 C+D), the R275W mutant has a behavior that is much more comparable to PINK1 dynamics than it does to YFP-Parkin (Fig. 5 A + B). Each pulse is followed by a recovery period in which Parkin levels return to statistically comparable levels as time zero. When compared side by side, it is evident that the R275W is being retained at the mitochondria far less than YFP-Parkin (Fig. 5E). An analysis of the second peak as a function of the first peak revealed that YFP-Parkin showed a significant increase compared to R275W (Fig. 5F).

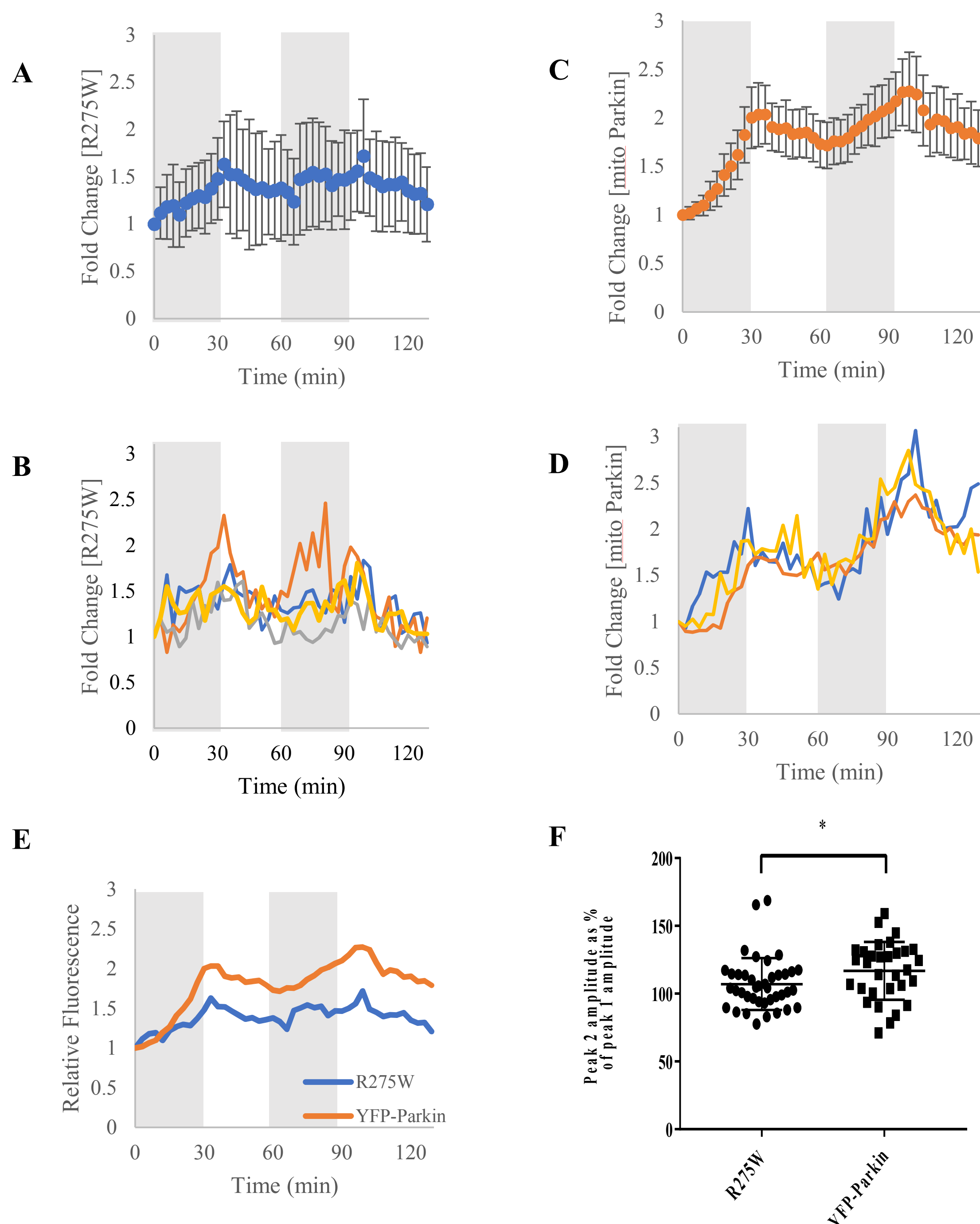

Figure 5. YFP-parkin and the R275W mutant show two distinct responses Hela cells transfected with YFP-parkin or the R275W construct were exposed to 30 minute pulses of CCCP. 30 minute exposures to these uncouplers, represented as gray areas, were followed by thirty minute recovery periods. The R275W mutant (A) does not increase in levels following multiple bouts of CCCP exposure whereas YFP-parkin (B) does. These two types of behavior are more evident in the single cell data, which shows a return in R275W to base levels (C) and building of YFP-parkin levels with each exposure (D). The difference in the two data sets is clear when plotted on the same graph (E). A peak analysis revealed a significant difference when comparing the second peak as a percentage of the first peak ($p < 0.05$). Data is from three biological repeats with a minimum of 30 cells per condition. Error is represented as S.E.M.

3.5 Validation of Results Using the Mitochondria-Specific Ionophore, BAM15

When studying the PINK1:Parkin pathway, most labs utilize the mitochondrial uncoupler CCCP (Kane et al., 2014; Okatsu, et al., 2012; Akabane, et al., 2016), as is the case in our lab as well (Bowling et al., 2019). CCCP, while very effective as a mitochondrial uncoupler, will also depolarize the plasma membrane and can interfere with autophagy-related processes. BAM15 is a recently developed mitochondria-specific uncoupler that reportedly does not cause the same side effects as CCCP, whilst accomplishing the same effect (Kenwood et al., 2013). To verify that the PINK1 and Parkin responses reported in this thesis were a consequence of mitochondrial depolarization and not off-target effects of CCCP, key experiments were repeated using BAM15 instead of CCCP.

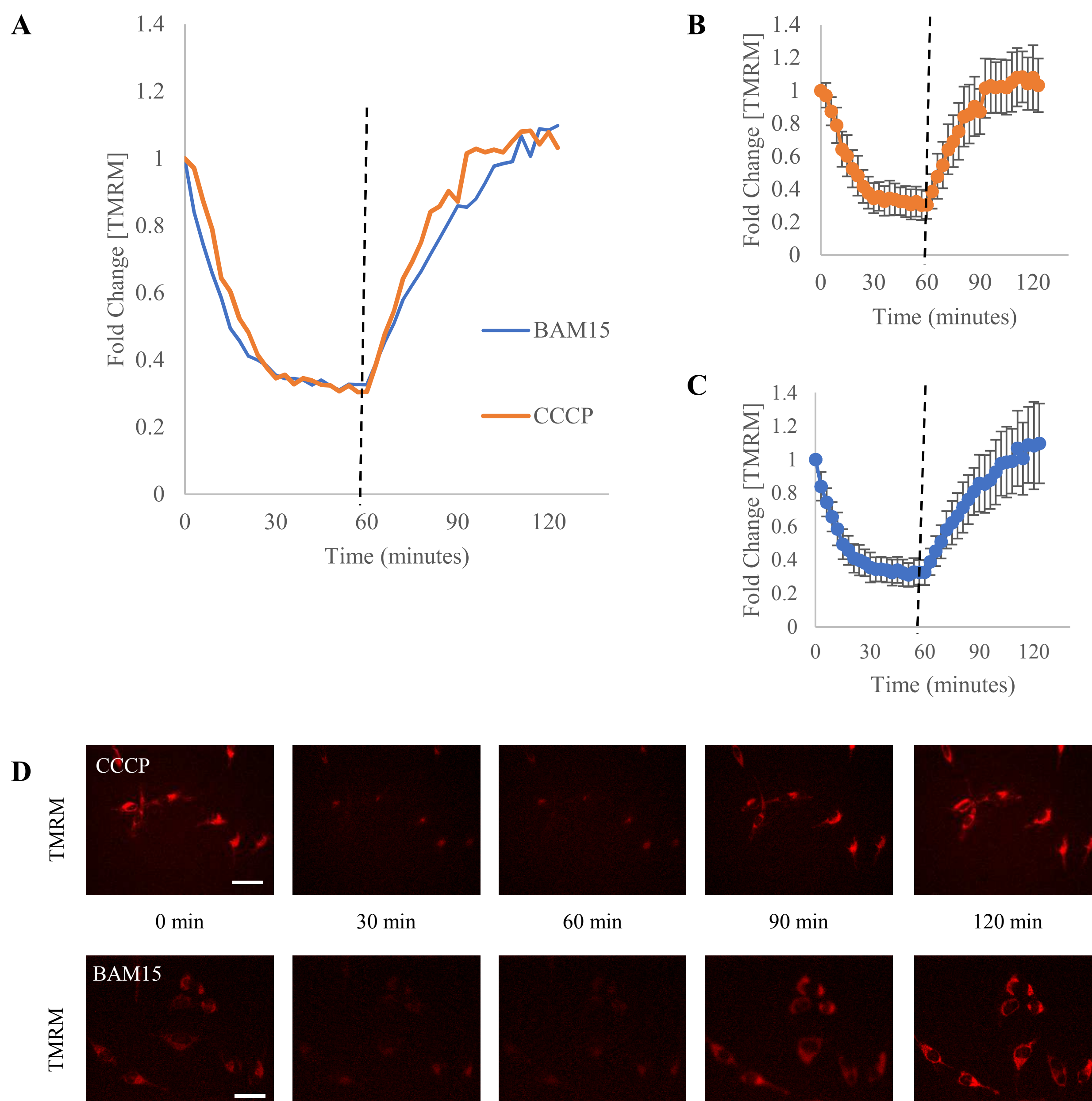

Figure 6. BAM15 elicits a comparable depolarizing effect to CCCP HeLa cells treated with TMRM were exposed to a 10 µM dosage of either CCCP or BAM15. Doses of mitochondrial uncouplers were administered at 0 minutes and washed out at 60 minutes. BAM15 and CCCP cause the same level of mitochondrial membrane depolarization (A) and are capable of a total recovery of MMP following removal of these compounds (B & C). The effects of these uncouplers are apparent in the live microscopy images, showing a clear pattern of loss of MMP and subsequent recovery (D). Scale bars represent 50 µm. Data is from three biological repeats with a minimum of 30 cells per condition. Error is represented as S.E.M.

To first assess the dosage of BAM15 required to cause a complete loss of MMP, HeLa cells were loaded with 10 nM Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM), which is a dye that fluoresces in mitochondria with an intact membrane potential. The cells were observed by fluorescence microscopy while incubated with either 10 μM CCCP or BAM15 for 60 minutes followed by a 60 minute recovery period. Similarly to CCCP, it was found that 10 μM BAM15 was sufficient to cause a complete loss of MMP and MMP was recovered at a similar rate on BAM15 washout (Fig. 6).

After establishing the dosage, BAM15 was utilized to replicate the YFP-Parkin high frequency pulse experiments. A comparison between the two quickly reveals the response to the two uncouplers is identical, showing virtually the same response when compared side by side (Fig. 7).

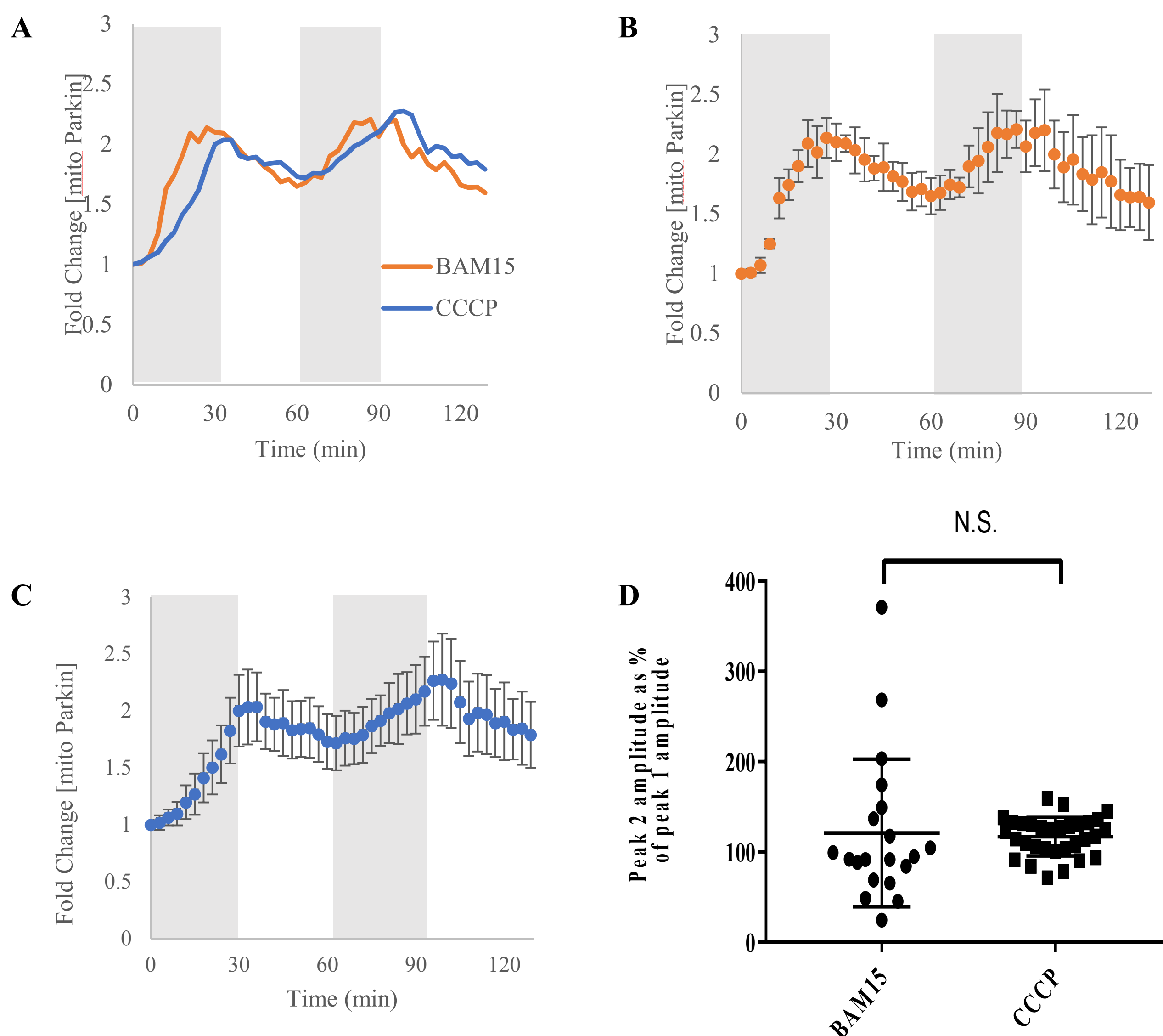

Figure 7. BAM15 and CCCP cause a similar parkin response Hela cells transfected with YFP-parkin were exposed to either 30 minute pulses of CCCP or BAM15. 30 minute exposures to these uncouplers, represented as gray areas, were followed by thirty minute recovery periods. The response of parkin is comparable when comparing the BAM15 and CCCP data (A). Both BAM15 (B) and CCCP (C) cause parkin levels to increase with each exposure, and show only slight decreases in parkin levels following washout. A peak analysis (D) revealed no significant difference when comparing the second peak as a percentage of the first peak. Data is from three biological repeats with a minimum of 20 cells per condition. Error is represented as S.E.M.

DISCUSSION

The PINK1:Parkin pathway directs the removal of damaged or dysfunctional mitochondria by establishing a unique coating of Ser65 ppUb chains on the OMM of affected regions of mitochondrial networks that facilitates the recruitment of autophagy adaptors and the autophagic machinery. While superficially simple and involving a relatively small number of distinct components, the operation of the pathway and how it interprets differing levels and types of mitochondrial stress remains unclear.

The first protein recruited in this system, PINK1, has been shown to be capable of rapid association and dissociation from the mitochondrial membrane in response to changes in MMP (Nyugen et al., 2016; Bowling et al., 2019). In this sense, PINK1 essentially reports on the current MMP-status of mitochondria and provides a mechanism to rapidly interrupt or reset the mitophagy process if MMP has been restored before autophagy adaptors are recruited and isolation membranes established around mitochondria. Once Parkin has been shed from the mitochondria, any Parkin remaining at the mitochondria may, hypothetically, retain the ability to form conjugate additional polyubiquitin on to OMM substrates but it would not be able to stimulate ppUb formation. Therefore, no additional Parkin binding sites at the OMM could be created and any existing ones would slowly be erased through the action of mitochondrial deubiquitinating enzymes, like USP30. This protein's diminished effect on phosphoubiquitin suggests that the longer mitophagy proceeds, the more challenging it is to reverse.

While PINK1 levels closely track with MMP, Parkin and ppUb can serve as persistent markers of recent mitochondrial insults. This behavior is evident in the high frequency pulsing experiments (Fig. 1) wherein PINK1 levels return to basal levels upon removal of CCCP but Parkin levels increase upon each subsequent exposure to CCCP, building in almost a stepwise fashion (Fig. 3+7). The low frequency pulsing experiments, however, show that while Parkin is retained at the mitochondrial surface for much longer than PINK1, it eventually dissociates from the mitochondrial surface, albeit at a much slower rate. These data as a whole make it very apparent that PINK1 and Parkin operate on two different time scales, ensuring both that there is an immediate response to changes in MMP and that prior changes in MMP, if relatively recent, are integrated as part of the current response.

Previous studies have established that Parkin utilizes ppUb as a docking site (Shiba-Fukushima et al., 2014). The binding of Parkin to ppUb is followed by a conformational change that allows for phosphorylation of the Parkin Ubl domain by PINK1 and, as a consequence, full activation of Parkin E3 ligase activity. As Parkin conjugates more ubiquitin chains to the surface of the mitochondria, PINK1 continues to phosphorylate both these ubiquitin moieties as well as new Parkin proteins recruited to the OMM. These relationships constitute interlocking feedforward and positive feedback loops (Bowling et al., 2019). If this model is correct, stopping the process of ubiquitylation of the outer mitochondrial membrane proteins by Parkin should prevent the long-lasting Parkin retention at the mitochondrial membrane following the reestablishment of MMP.

To test this hypothesis, a Parkin mutant that still retained its ability to be recruited to the OMM but lacked the ability to direct ubiquitin chain formation had to be identified. The R275W mutant fit these criteria, whilst both having comparable kinetics to YFP-Parkin and being relatively easy to clone with only the introduction of a single point mutation required.

Upon repolarization, the dynamics of the R275W mutant dissociation from mitochondria is markedly different than that of YFP-Parkin. Although there is no significant difference between the two data sets when comparing the final time point, it is obvious that there is a difference in how these two proteins respond when considering the dissociation kinetics following the removal of CCCP (Fig. 4). This difference in kinetics is more apparent when considering the high frequency pulsing experiments in which the R275W mutant levels do not increase following multiple bouts of 10 μM CCCP exposure (Fig. 5). The R275W mutant's inability to polyubiquitinate substrates results in Parkin levels decreasing relatively quickly once MMP increases, likely due to the lack of sufficient Parkin docking sites. Notably, not all of the cells follow the same type of response. While many of the cells dissociate at a rate that is far more rapid than that of the typical YFP-Parkin there are still some cells that show very little Parkin dissociation following the removal of CCCP. This difference in behaviors is particularly noticeable when comparing the averaged data to that of the single cell data. While the dissociation of R275W is clear in the single cell data, it is far less apparent when looking at the averaged data. A previous study suggested that mutants within either of the RING finger domains have the potential to alter Parkin ppUb binding affinity (Sriram et al., 2005). While many of the mutations they observed reduced Parkin's binding ability, there were

several mutations that had a higher propensity to bind to certain proteins. Among these was the R275W mutant, which was noted to have an increased affinity for synphilin-1. This study proposed the idea that, while most Parkin mutations imbue the protein with an inability to initially bind substrates, some, such as R275W, could be altered in such a way that they are unable to effectively release substrates. Taking this into consideration reveals the possibility that R275W is being retained at the mitochondrial membrane due to an inability to unbind from membrane substrates. This would explain why the difference between YFP-Parkin and R275W is statistically insignificant when considering the one hour pulsing data.

In addition to determining the effects of changing the frequency of pulsing, we also wanted to understand the effects that partial membrane depolarization would have on PINK1 recruitment over a long time scale. PINK1 mounts a response to both a total and a partial loss of MMP. When there is a sustained, complete loss of MMP, PINK1 levels increase, and fluctuate in levels, but do not return to a low-level state in the cell. However, when there is a partial loss of MMP, PINK1 levels increase initially, but slowly decrease to basal levels over time (Fig. 2). PINK1's response to the 10 μM dosage makes sense in that a sustained, complete loss of MMP would prevent PINK1 degradation as it would not be cleaved in the mitochondrial matrix. A 5 μM dosage appears to be enough to elicit a PINK1 response, but not one that is sustained over time. A 5 μM dosage may warrant a PINK1 response but does not decrease MMP enough to be sustained over time suggesting that 5 μM CCCP may be on the cusp of a critical threshold where PINK1 levels are sustained over time. It could also be the case that, at this level of depolarization, PINK1 levels naturally fluctuate over time, mounting routine responses

over time. This would require that PINK1's behavior is observed on a longer time scale to make any conclusions. As an alternative hypothesis, CCCP may not be stable for long periods of time under tissue culture conditions. Its slow degradation over the course of 24 h may result in the slow increase in MMP and PINK1 loss.

The implications of such types of damage are especially relevant when considering that the non-inherited form of PD is often associated with increased levels of respiratory chain complex I deficiency. While the role this deficiency directly plays in PD is unclear, it has been noted that such deficiencies cause a partial loss of MMP evidenced by TMRM experiments (Distelmaier et al., 2009). Furthermore, impaired complex I activity has been correlated to an increase in oxidative stress as a byproduct of increased ROS production. Oxidative stress can be even more potent in dopaminergic neurons that are already prone to the generation of ROS as a byproduct dopamine metabolism (Chinta and Andersen, 2005).

While it is commonplace to utilize CCCP as an uncoupler, some of these results were replicated with a different mitochondrial uncoupler as a control. The results showed that BAM15 generated a response that was almost indiscernible from CCCP (Fig 7A). The results of the BAM15 experiments suggest that the response generated by CCCP is genuinely due to a loss of MMP.

These findings show that both partial losses of MMP and recurring, total losses of MMP each generate a unique response from the mitophagy pathway that the current literature does not address. These data support that, while PINK1 acts as a reporter of

MMP, Parkin (utilizing ubiquitin as a docking site) can act as a marker for recent mitochondrial damage.

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