Elucidating the molecular mechanisms that control the switch between mitochondrial repair and destruction after mitochondrial damage

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Elucidating the molecular mechanisms that control the switch between mitochondrial repair and destruction after mitochondrial damage

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Abstract

Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic neurons from the substantia nigra pars compacta, resulting in tremors at rest, bradykinesia, stiffness of muscles, retropulsion, and poor balance. A leading hypothesis on the cause of PD is dysfunction of the mitophagy pathway. Mitophagy is a mitochondrial quality control pathway that destroys dysfunctional mitochondria. Currently, it is unclear how this pathway responds to minor or transient impairment of function. Here we show that intermediate doses of the mitochondrial poison, CCCP, cause partial depolarization of mitochondria and transient association of PINK1 proteins with the outer mitochondrial membrane (OMM) lasting ~60-90 minutes. Our data suggests that this mechanism enables the cell to 'abort' the mitophagy process if mitochondrial function is restored, preventing inappropriate destruction of healthy mitochondria. These new insights into PINK1 function may inform future studies that seek to enhance or maintain mitochondrial health in PD patients.

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Introduction

Background

Parkinson's disease (PD) is a neurodegenerative disease that affects around one million people in the United States. The characteristic symptoms of PD are olfactory dysfunction, tremors at rest, bradykinesia, stiffness of muscles causing hypomimia, retropulsion, and poor balance, and sometimes dementia (Shulman *et al.* 2011 and Hawkes *et al.* 1997). Degeneration of dopaminergic neurons (DA neurons) in the substantia nigra pars compacta (SNpc), a section of the brain involved with motor control, prevails in PD. SNpc neurons contain large, highly branched, unmyelinated axons that require large amounts of energy to function properly. Perhaps due to their energy requirements, SNpc neurons are more susceptible to biological stresses that impact upon normal mitochondrial function and these stresses will often promote the apoptosis of DA neurons (reviewed in (Pissadaki and Balam 2013).

In healthy brain tissue, mitochondrial health is maintained through the action of the mitophagy pathway, a mitochondrial 'quality-control' mechanism that destroys defective or damaged mitochondria before they can compromise the health of the cell's entire mitochondrial network (Tatsuta and Langer 2008). The autosomal inheritance of defective copies of genes encoding key mitophagy proteins (such as parkin (PARK2) and PTEN-induced putative kinase 1 (PINK1; PARK6)) can lead to the development of PD (Schulte *et al*. 2011).

PINK1 surveils the mitochondrial network and becomes stably associated with non-functioning, depolarized regions (Narendra *et al.* 2010A). After it has associated with the outer mitochondrial membrane, PINK1 recruits Parkin. Recruitment of parkin leads to the polyubiquitination of multiple mitochondrial proteins (Sarraf *et al*. 2013) and the subsequent destruction of the damaged mitochondria by the autophagy system (Nerendra *et al.* 2008). However, our lab and other groups have observed that the mitophagy process can be 'aborted' if the mitochondrial damage can be repaired, and the normal polarization of the mitochondrial membranes is restored (Zhang *et al.* 2014). However, it is currently unclear exactly how the mitophagy system 'decides' which damaged mitochondria are beyond repair and should be destroyed and which are salvageable and should be repaired.

Literature review

Mitochondrial dynamics and the mitophagy pathway

Cellular stressors and poisons can compromise mitochondrial function (e.g. oxidative phosphorylation) by depolarizing mitochondrial membrane potential. These mitochondrial insults allow for proton diffusion across the inner mitochondrial membrane without it being coupled to ATP synthesis through the electron transport chain. This can result from the stressors either causing damage to ATP-synthase or increasing the permeability of the inner mitochondrial membrane (Sack 2006). The depolarization of the inner mitochondrial membrane and the hindering of its ability to produce ATP is what determines mitochondrial dysfunction in the context of this paper.

The health of a cell's mitochondrial network, which is vital for its ongoing survival, is controlled by a series of mitochondrial quality control (MQC) systems, such as the mitophagy pathway. This pathway constantly surveils the mitochondrial network, identifying regions of damage and regulates the repair or removal of these regions by the autophagy system (Kim *et al*. 2007). In this way, the mitophagy system prevents the catastrophic release of pro-apoptotic elements from critically damaged mitochondria (Maiuri *et al*. 2007). At its core, the pathway consists of PINK1 and Parkin proteins, a mitochondrial serine/threonine protein kinase and E2/3 ubiquitin ligase, respectively (reviewed in (Yang and Yang 2011)). Under normal circumstances, PINK1 is a highly labile protein that is imported and cleaved by a mitochondrial protease, the identity of which remains controversial but is suspected to be presenilins-associated rhomboid-like protein (PARL; Deas *et al.* 2010). However, at damaged, depolarized mitochondria, PINK1 accumulates on the surface of the organelle. Once there, it recruits Parkin to the mitochondria, activating it by phosphorylating the amino-terminal ubiquitin-like domain (Kim *et al.* 2008). Activated Parkin is then able to polyubiquitinate a range of different mitochondrial surface proteins (Sarraf *et al.* 2013) initiating the proteasomal degradation of some proteins such as mitofusins 1 and 2 (Chan *et al.* 2011) which initiate the fragmentation of damaged regions of the mitochondrial network (Gegg *et al.* 2010). Other proteins acquire linear ubiquitin chains which act as adaptors to recruit the autophagic machinery that ultimately destroys these labeled mitochondria (Narendra *et al.* 2010B). Over the past year, it has been confirmed that PINK1 directly phosphorylates ubiquitin at the serine 65 region in a parallel fashion to Parkin phosphorylation within the ubiquitin-like domain of Parkin (Kane *et al.* 2014). Phosphorylated Parkin and PINK1 work in

tandem, as a feed-forward mechanism, to produce phosphorylated K63-linked ubiquitin chains that act as docking platforms for autophagy receptors such as OPTN and NDP52 leading to mitochondrial destruction (Koyano *et al.* 2014, Lazarou *et al.* 2015, and Ordureau *et al.* 2015). While it is possible for PINK1 to stimulate autophagy adaptor recruitment in the absence of Parkin, this occurs at a significantly impaired rate illustrating the importance of the PINK1:Parkin relationship in mitophagy (Matsuda and Tanaka 2015).

Mitophagy vs. repair

Recently, a new mechanism has been discovered in the MQC pathway. Depending on the nature of the mitochondrial stressor, PINK1-Parkin acts as a 'damage-gated molecular switch' determining whether apoptosis, mitophagy, or repair occurs (Zhang *et al*. 2014). However, the molecular mechanism that grants PINK1-Parkin the ability to produce these distinct, stimulus-specific responses has yet to be fully elucidated. It is also unclear whether the proteins, PINK1 or Parkin, are actually involved in mitochondrial repair, but they may work in parallel with the process. Previous studies have only investigated the effects of damage that renders mitochondria completely defective (as defined by a complete loss of mitochondrial membrane polarization). These studies have not delved into how PINK1-Parkin responds to varied levels of a particular mitochondrial stressor. Mid to low range mitochondrial stresses causing only partial or transient decreases in mitochondrial membrane polarization may simply be ignored, or they may elicit a repair-like response. This unanswered question in the field will be the core focus of this study.

Techniques used to study mitophagy

To study the effects of mitochondrial dysfunction, controlled damage must be simulated. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a depolarizing agent, diminishes mitochondrial membrane potential in selected cells by inducing H^+ permeability to the mitochondrial membrane. The resulting loss of mitochondrial membrane polarization induces recruitment of PINK1 and Parkin to the membrane (Chan *et al.* 2011). CCCP may have limitations such as asynchronous and spatially uncorrelated initiation of mitophagy (Yang and Yang. 2011), but it is appropriate for use in simple, cell culture models of mitophagy, and in previous experiments conducted by the Nelson lab, titration of CCCP allowed for the identification of specific doses that created either moderate (5 μM) or complete (>10 μM) mitochondrial membrane depolarization resulting in complete mitophagy of the mitochondrial network.

Visualizing and measuring mitophagy

To effectively visualize the effects of CCCP in mitophagy, a plasmid construct containing PINK1 fused with a fluorescent protein marker is needed. These plasmid constructs are produced from synthetic derivatives of bacterial extrachromosomal genetic material that are modified to express exogenous genes such as fluorescently tagged PINK1. Once formed, these plasmids are easily replicated in strains of *Escherichia coli* and can be modified using standard molecular cloning techniques (Maniatis *et al.* 1982). Plasmids can be easily transfected into mammalian cells to elicit the expression of desired exogenous genes (Pollard *et al*. 1998). Although plasmid gene expression is short-lived, this process is safer than others (e.g. lentiviral transduction) and allows for

fluorescently tagged PINK1 expression to be tracked and quantitatively measured through fluorescence microscopy to better understand its effects on biological systems, specifically mitophagy (Day and Schaufele 2008, Lippinscott-Schwartz and Patterson 2003, and Sung and McNally 2011).

Thesis Statement

"PINK1 operates as a 'biological filter' for the detection of mitochondrial stress levels, which is capable of both initiating the mitophagy process and 'aborting' it if mitochondrial function recovers."

This study examines the response of PINK1 proteins (as defined by the expression level and subcellular localization of fluorescently tagged PINK1 proteins) to varying levels of mitochondrial stress, as generated by dosing with different concentrations of the mitochondrial poison, CCCP. The observation of transient expression of PINK1 shown by fluorescently tagged PINK1 in conjunction with the mitochondrial polarity reporter, TMRM, supports the thesis statement and suggests that PINK1 initiates mitophagy and also acts as a "self-destruct" mechanism halting mitophagy when minor fluctuations in mitochondrial polarity move toward recovery.

Materials and Methods

Cell Culture and Reagents

The HeLa human cervical carcinoma cell line (HeLa; ATCC, USA) which has a high transfection efficiency and is Parkin null was utilized for this study. HeLa cells were maintained in Dulbecco's Modified Eagles's Medium (DMEM) augmented with heat inactivated 10% Fetal Bovine Serum (FBS), 0.1% penicillin (100 units/mL)/streptomycin (100 µg/mL), and 0.1% nonessential amino acids. The cells were grown to at least 80% confluence before being utilized. The cells were maintained in a humidified incubator at 37ºC supplemented with 5% carbon dioxide.

Molecular Cloning

The molecular cloning of pPINK1-mCherry was a multistage process. Primers (Table 1) were used to amplify the full-length human PINK1 (isoform 1) cDNA sequence from a pre-existing PINK1 expression vector (pCDNA3-PINK1, Nelson Lab).

Table 1. Sequence of DNA primers used to amplify the human PINK1 cDNA.

Sequence Name	Sequence 5' to 3'
EcoRI_PINK1_F	CGTTAGAATTCTTATGGCGGTGCGACAGGCGCTGG
BamHI_PINK1_R	CGTTAGGATCCGCCAGGGCTGCCCTCCATGAGC

Amplification with the reverse primer ablated the 3' stop codon enabling the in-frame fusion of PINK1 with the mCherry coding sequence (CDS). Restriction enzymes, *EcoR1* and *BamH1*, were utilized to produce cuts at the 5' and 3 ends of the PINK1 PCR product, respectively. The PINK1 PCR product and an empty pmCherry-N1 cloning vector were cut using both of the aforementioned enzymes simultaneously as a 'double digest'. The cut, linearized DNA was then electrophoresed on a 0.8% gel containing Ethidium Bromide (10 mg/mL). Vector and PINK1 fragments were purified from the gel using a QIAquick Gel Extraction Kit (Qiagen, NLD), in accordance with manufacturer's guidelines, and ligated using T4 DNA ligase (NEB, Ipswich, MA). The ligation products were transformed into competent *E. coli* (DH5α), and successful transformants were selected for by growth on kanamycin-containing LB agar plates (50 µg/mL). The resultant clones were then screened using restriction-digest based mapping techniques.

Transfection

Protein expression plasmids were transfected into HeLa cells using the lipid-based membrane destabilizing reagent polyethyleneimine (PEI). Production of PEI involved reconstituting lyophilized PEI (Polysciences) in sterile endotoxin-free water to a concentration of 1 µg/µL, gently heating the mixture until the PEI entered into solution, and, lastly, adjusting the pH to 7.2 with HCl. Solubilized PEI was sterilized by passing it through a 0.22 µm syringe filter. PEI solutions were stored at -80ºC prior to use.

Transfections required a mixture of 100 µL of serum-free media and 3 µL of PEI for every microgram of DNA to be delivered to the cells. Typically, 1 µg of DNA was required per $1x10^5$ cells. Therefore, when preparing cells for imaging in 35mm glass-

bottom (MatTek) dishes, $1x10^5$ cells were plated and transfected the following day with 1 µg of DNA. To prepare 60 mm tissue culture dishes of cells for western blotting experiments, $3x10^5$ cells were plated and transfected the following day with 3 µg of DNA. For both plate sizes, serum and antibiotic-free media and PEI were combined together and vortexed for ten seconds before plasmid DNA was added. The solution was incubated for fifteen minutes at room temperature and then added to the cells. The transfection mixture was incubated with the cells for 6 hours and later removed to minimize the cell toxicity effects of PEI.

Western Blotting

HeLa cells cultured to 80% confluence in 60 mm tissue culture plates were transfected and treated with the indicated reagents 24 hours post transfection. After the required incubation period, cells were lysed in Laemlli lysis buffer (1.25 mL 0.5 Tris-HCl, at a pH of 6.8, 1 mL of glycerol, 2 mL of $10\%_{(w/v)}$ SDS, and 250 µL $0.1\%_{(w/v)}$ Bromophenol blue and the final volume is brought up to 10 mL with DI H_2O) and boiled for 10 minutes to destroy genomic DNA and linearize proteins. Proteins extracted from transfected HeLa cells were separated by electrophoresis on 8% SDS-PAGE gels and transferred to nitrocellulose membranes which were blocked with 5% BSA in TBS/T. The membranes were incubated overnight with the appropriate primary antibodies (raised against either PINK1 (1:1000) or Actin (1:5000, control)), washed in 3 changes of TBS/T, and incubated with the appropriate HRP-conjugated secondary antibodies (i.e. anti-mouse-HRP to detect mouse-raised primary antibodies, 1:2000). Probed membranes were visualized with a

home-made ECL solution using a ChemiDoc^{TM} MP Imaging System and Imaging Lab software (Bio-Rad).

Time-lapse Fluorescence Imaging

HeLa cells were plated in 35 mm glass-bottom (Cellvis, USA) dishes at $2x10^5$ cells in 2 mL of media and imaged with 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). The microscope was controlled using Nikon Elements Software (Nikon, USA).

The cells were transfected a day before imaging and exposed to specified levels of CCCP after fields were selected. Images were taken at 3-minute intervals for the indicated periods of time. EGFP was excited through a 465-495 nm excitation filter and emitted light was detected through a 515-555 nm barrier filter, reflected from a 505 nm dichroic mirror. mCherry was excited through a 535-550 nm excitation filter and emitted light was detected through a 610-675 nm barrier filter, reflected from a 565 nm dichroic mirror.

To perform 'pulsing' experiments, small molecule inhibitors of mitochondrial function (i.e. CCCP) were added to the cells in complete, pre-warmed culture medium for the indicated periods of time and then removed. The cells were washed with at least 3 changes of pre-warmed medium (without inhibitors) and fresh, warm culture medium was added before resumption of the live cell imaging. Typically, this pulsing process took no longer than 2 min and therefore could take place between programmed imaging cycles (i.e. image acquisition at 5-minute intervals).

Image Analysis

After image acquisition, 14-bit nd2 images were exported and analyzed using FIJI (Schindelin *et al.* 2012). In brief, Regions of interest (ROI) were selected by drawing polygons around cells expressing the desired fluorescent proteins or reagents. Integrated intensities were measured by adding up each pixel's fluorescence intensity inside of the ROI Background fluorescence was subtracted from the mito-mCherry and PINK1-EGFP fluorescence signals from individual cells, recorded as the integrated intensity from the entire area of the cell. All intensity readings were normalized to their initial T = 0 min values (i.e the recorded fluorescence signal from each cell from the first recorded frame of each experiment). Three independent biological repeats of each experiment were performed.

Results

Production of a plasmid construct to express PINK1-mCherry fluorescent fusion proteins in mammalian cells

In order to visualize the stabilization and translocation of PINK1 proteins to the mitochondria (the initiation step of the mitophagic process), a plasmid construct to express chimeric fusions of PINK1 with mCherry, a red fluorescent protein derived from corals, was produced. After construction, this plasmid could be introduced into mammalian cell lines, such as the HeLa cervical carcinoma cell line, by transfection. The mCherry sequence was fused to the C-terminus instead of the N-terminus of PINK1 because the N-terminus houses the mitochondrial-targeting sequence (MTS) (Okatsu *et al.* 2015). The MTS is essential for PINK1 trafficking to the mitochondrial membrane during mitophagy and this could possibly be inhibited by or blocked by conjugating a large fluorescent protein such as mCherry to this region of the protein. Furthermore, in normal, undamaged mitochondria, the N-terminal MTS sequence is cleaved off by a mitochondrial protease. This would cause the mCherry tag to dissociate from the core PINK1 protein, which would prevent us from accurately tracking PINK1 levels and localization in cells.

In brief, the cloning process to produce pPINK1-mCherry (shown in Figure 1A) involved the ligation of the human PINK1 cDNA sequence (amplified by PCR from a PINK1 CDS-containing expression vector) into an empty pmCherry expression vector. When the ligation products were transformed into competent bacteria, we obtained multiple clones. We screened five of these clones to test whether or not they contained the PINK1 sequence. This was performed by extracting the plasmid DNA from the clones and

subjecting small samples of this DNA to a diagnostic digest with *Eco*RI and *Bam*HI restriction endonucleases. The digested DNA was electrophoresed on a 0.8% agarose gel to separate the digested DNA fragments by size, enabling us to discriminate between correct and incorrect clones. The first gel (Figure 1B) contained a control lane with empty pmCherry cut with the same enzymes as the pPINK1-mCherry clones. Clones one through five of pPINK1-mCherry each displayed two distinct bands on the gel. One band for each of the clones matched directly with the cut pmCherry control (~6157 bp) while the remaining bands were at equal positions down the gel and matched with the expected size of the PINK1 cDNA sequence (~2700 bp).

One of the 5 clones was selected for further analysis. This involved electrophoresing a sample of *Eco*RI and *Bam*HI cut clone on a 0.8% agarose gel against PINK1 DNA, amplified by PCR from pcDNA3-PINK1, which contains the full-length human PINK1 cDNA sequence (Figure 1C). The lane housing the known PINK1 PCR product exhibited only one band at (~2700 bp), as expected. The other lane containing the cut plasmid DNA from the selected clone displayed two fragments, one matched the known size for the pmCherry plasmid and the other matched the control PINK1 fragment, confirming the identity of our clone.

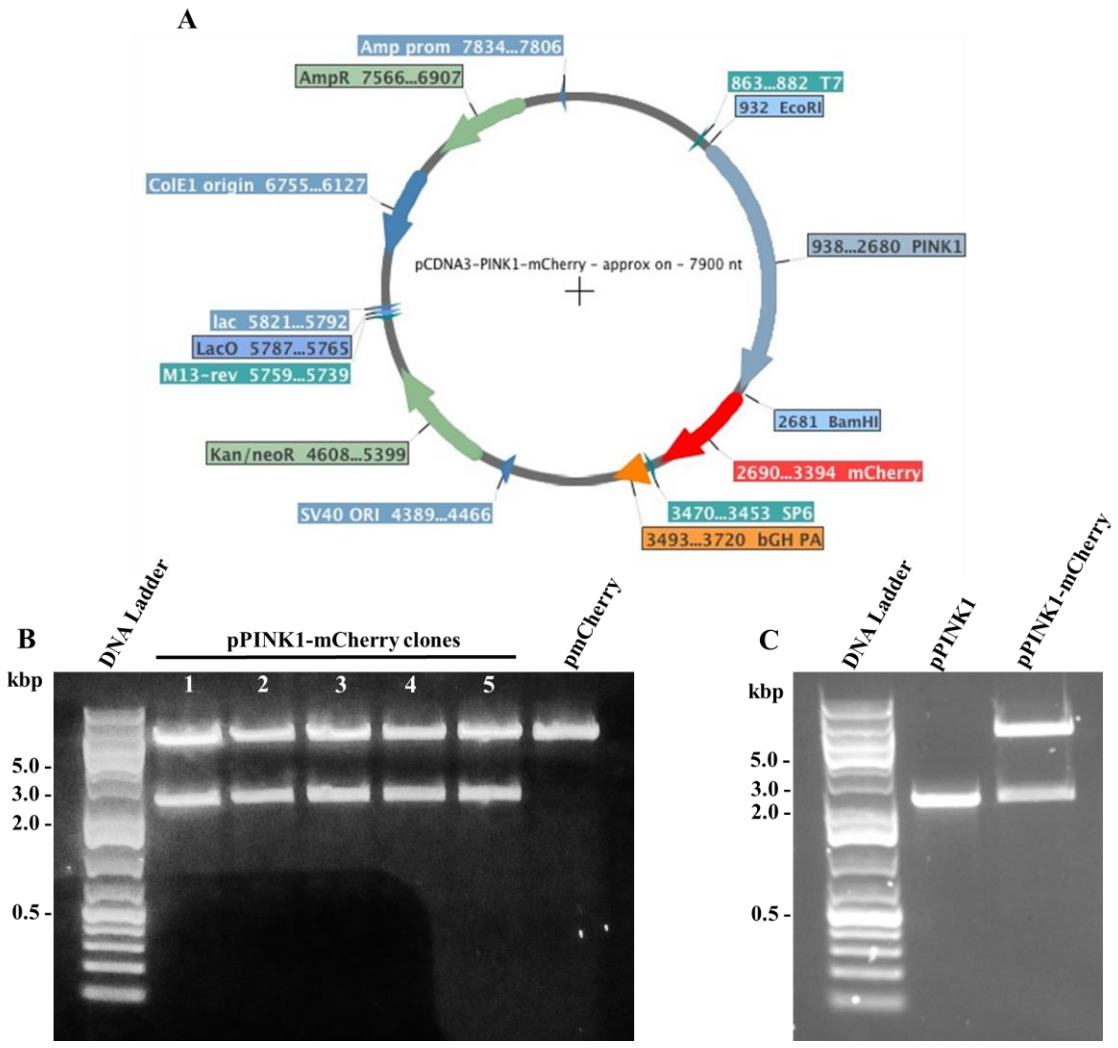

Figure 1. Production of a plasmid construct to express fluorescent fusions of PINK1 and mCherry in mammalian cells. A. Approximate plasmid map of pPINK1-mCherry. The orientation, position, and relative sizes of PINK1 and mCherry are representative of the true plasmid structure. *Eco*RI and *Bam*HI restriction endonuclease cut sites used to clone PINK1 into the pmCherry expression vector are also presented on the map. **B.** Screening of 5 puative pPINK1-mCherry clones by double restriction digest with *Eco*RI and *Bam*HI endonucleases. Similarly cut empty pmCherry is also represented on the 0.8% agarose gel as a control. All five clones displayed two bands (~2700 bp and ~6157 bp) as was expected for the correct pPINK1-mCherry construct. **C.** One of the pPINK1-mCherry clones cut with *Eco*RI and *Bam*HI and tested against PCR amplified PINK1 cDNA. The pPINK1-mCherry clone separated into two bands with one band matching with the full length PINK1 cDNA (~2700 bp).

Validation of PINK1-mCherry in HeLa cells by western blotting

Having successfully produced a plasmid construct to express PINK1-mCherry proteins, it was vital to determine whether proteins expressed from this plasmid in cells were (i) expressed as a chimeric fusion and (ii) were stabilized like untagged PINK1 in response to depolarization of the mitochondria. This was performed by transfecting the pPINK1-mCherry or the pcDNA3-PINK1 constructs into HeLa cells, which would express mCherry tagged and untagged versions of the PINK1 protein, respectively. These cells were treated with CCCP for one hour to depolarize the mitochondria and induce mitophagy, lysed to extract the proteins, and then subjected to western blot analysis using anti-PINK1 antibodies to determine the size and the expression level of PINK1 in the cells. If the plasmids operated as required, we expected that we would observe bands at ~91.8 kDa for the full-length PINK1-mCherry fusion. Untagged PINK1 would produce bands at ~63 kDa. We also expected to see an increase in the levels of the full-length PINK1 protein after CCCP treatment, as the protein is stabilized. As predicted, we observed that PINK1-mCherry had a higher molecular weight than its untagged counterpart and both untagged PINK1 and PINK1-mCherry levels in HeLa cells increased in response to CCCP treatment, indicative of the expected stabilization (Figure 2). The Actin control displayed consistent loading of each sample. As the chimeric fusion protein was stabilized like the untagged protein, these data suggested that the pPINK1-mCherry construct would be suitable for the measurement of the kinetics of the PINK1 response to mitochondrial depolarization in live cells.

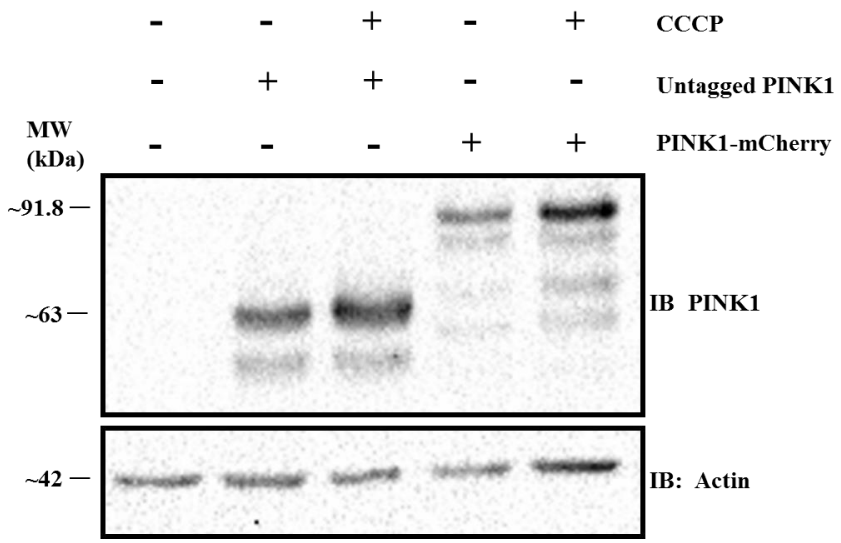

Figure 2. Validation of PINK1-mCherry fusion proteins by Western Blotting. Anti-PINK1 Western Blotting of whole cell protein lysates from HeLa cells expressing PINK1 or PINK1-mCherry incubated with or without CCCP for one hour. Protein lysates were also western blotted for actin as a loading control.

Effect of increasing CCCP doses on mitochondrial membrane potential

To determine whether the kinetics of the PINK1 response to mitochondrial stress differ when different levels of mitochondrial stress are experienced, we needed to develop a method to reproducibly induce different levels of mitochondrial stress in cells. In order to achieve this, we titrated CCCP, an inhibitor of mitochondrial oxidative phosphorylation onto cultured HeLa cells. Three doses of CCCP (2.5, 5, and 10 µM) were used to depolarize the mitochondrial membrane polarity (MMP). We expected that increased concentrations of CCCP would result in larger decreases in MMP. This was measured using TMRM, a fluorescent dye that only stains polarized mitochondria. In cells treated with TMRM, TMRM fluorescence is proportional to MMP and mitochondrial function. As

illustrated in Figure 3, we observed that MMP decreased as CCCP dose increased (0 – 10 µM). The control group, which was not treated with CCCP, exhibited insignificant changes in fluorescence over a two hour period while 2.5, 5, and 10 µM CCCP treated cells showed decreased levels of fluorescence of approximately 75%, 50%, and 25%, respectively (Figure 3A). However, individual HeLa cells exposed to 5 and 10 µM CCCP doses exhibited markedly varied TMRM responses, often exhibiting transient fluctuations in TMRM staining post CCCP until a new steady-state of TMRM fluorescence was achieved. High levels (10 µM) of CCCP induced a nearly complete loss of TMRM signal in cells while mid-range doses (5 µM) induced a slower, more modest drop in TMRM signal, often with a slight increase in signal by the end of the time course and/or other fluctuations (Figure 3B). Based on these data, it could be concluded that different doses of CCCP can be used to produce discrete levels of MMP that could be used to study the responses of PINK1 to different levels of mitochondrial stress or damage. A comparison of the results revealed that each dose created distinctly different MMP levels (Figure 3C) leading to the assumption that each dose would produce different effects in treated cells (e.g. PINK1 association and dissociation from the outer mitochondrial membrane).

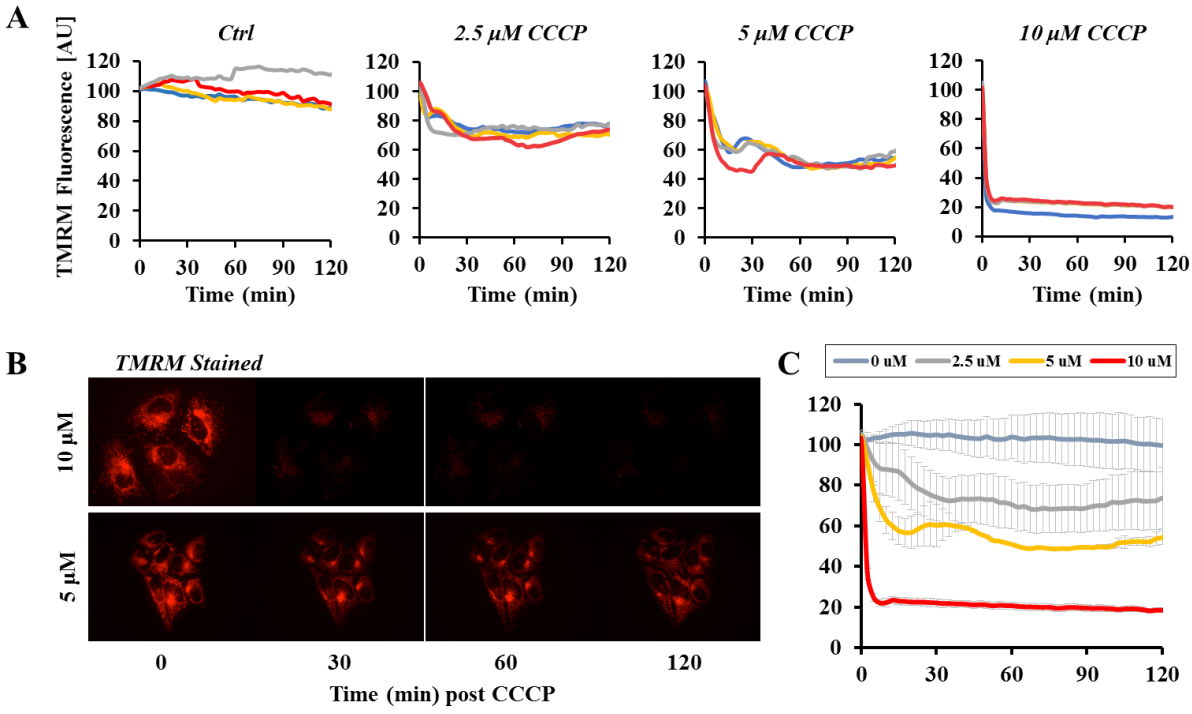

Figure 3. Modulation of mitochondrial membrane polarization by titration of CCCP. **A.** MMP, as reported by TMRM fluorescence in TMRM-loaded HeLa cells treated with different concentrations of CCCP. TMRM fluorescence was measured by live cell fluorescence imaging for 2 h immediately following CCCP addition. A decrease in MMP occurs as CCCP concentration increase (left to right). TMRM fluorescence data is presented for 4 representative cells per condition. **B.** HeLa cells incubated with TMRM at 37ºC for 15 min treated with 10 µM (top images) and 5 µM CCCP (bottom images). **C.** Average TMRM fluorescence over time for cell populations treated with the indicated concentrations of CCCP (0, 2.5, 5, and 10 µM). Error bars represent SEM. All data presented in this figure was gathered and analyzed by J. Logan Bowling (Nelson Lab, MTSU, 2015).

Divergent effects of different CCCP doses on the PINK1 response to mitochondrial depolarization

Having established the effect of discrete CCCP doses (0, 2.5, 5, and 10 µM) on MMP, we were then able to measure the effect of these doses on (i) the morphology of mitochondria and (ii) the association and dissociation of PINK1 from the outer mitochondrial membrane by transfection of HeLa cells with pShooter-Mito-mCherry, which encodes a red fluorescent marker of the mitochondria, and pPINK1-EGFP, encoding a green fluorescent fusion of PINK1, which was produced in exactly the same way as pPINK1-mCherry. For these experiment, pPINK1-EGFP was utilized instead of pPINK1-mCherry as the green fluorescence from PINK1-EGFP can be distinguished from the red fluorescence of the mitochondrial marker.

From past experimentation in the Nelson lab, we expected that cells exposed to high doses of CCCP (i.e. 10 µM) would exhibit rapid and long-lasting accumulation of PINK1 at the mitochondria accompanied by the fission of the mitochondrial network into small units. Prior to this study, we had not measured the PINK1 response to lower CCCP doses. However, we predicted that cells treated with 2.5 µM CCCP would show little to no mitochondrial PINK1 accumulation and that 5 µM CCCP-treated cells may display transient attachment of PINK1 to the mitochondria.

When control cells that were not exposed to CCCP were imaged by live cell microscopy for 3 hours no increase of association of PINK1-EGFP to the OMM was observed (Figure 4A and B). Furthermore, mitochondria networks constantly reorganized throughout the time course but remained largely fused as long mitochondrial strands rather

then smaller mitochondrial units or fragments which are indicative of the early stages of mitophagy (Figure 4A).

Treatment with 2.5 µM CCCP displayed little change in PINK1-EGFP association to the OMM (Figure 4C), but some cells experienced small, transient increases in PINK1 levels within the first 20 min post-CCCP treatment. As for morphological response, the mitochondria began to retract towards the nucleus (t = 30 min) but later exhibited normal morphology as time progressed.

Cells exposed to 5 µM CCCP displayed transient expression of PINK1-EGFP at the OMM. PINK1 fluorescence increased up until 60-90 min post CCCP and then rapidly dissociated from the OMM. The timing of this dissociation varied between individual cells. The morphology of the mitochondrial network experienced a similar, and reversible change. Mitochondria began to fragment, forming small ring-like structures (t = 30 to 60 min), known as 'mitochondrial donuts' but began to re-fuse once PINK1 had dissociated from the OMM (t = 90 to 120 min) (Figure 4E). The sharp peak and rapid decline seen in the quantification of PINK1 fluorescence was surprising and has not been previously reported in the mitophagy literature (Figure 4F, t = 60). At this stage in the investigation, it was not clear how this might be produced.

Lastly, treatment with 10 µM CCCP was accompanied by a stable and seemingly irreversible increase in PINK1 levels at the OMM in most cells. The mitochondrial network also retracted towards the nucleus, fragmenting into the mitochondrial donuts as was observed in cells treated with 5 µM CCCP, but these mitochondria did not recover (Figure 4G), and PINK1-EGFP fluorescence increased steadily over time with no hint of decrease (Figure 4G and H).

A semi-quantitative overview of the PINK1 response in cell populations treated with each CCCP dose (2.5, 5, and 10 μM) suggests that different levels of mitochondrial stress produce distinct outcomes (Figure 5). Cells treated with low levels of CCCP (2.5 μM) were unlikely to exhibit an increase of PINK1 expression and those that did rarely showed prolonged PINK1expression. Mid-level CCCP treatment (5 μM) displayed almost equal opportunity for either no expression or transient expression of PINK1-EGFP with a small number of cells showing prolonged expression. Lastly, high levels of CCCP (10 μM) resulted in prolonged expression in most cells with few or no cells showing transient or no increase in PINK1 levels.

These experiments illustrate the three potential outcomes of the PINK1 response to mitochondrial damage and display the ability of different doses of CCCP to control mitochondrial polarity and initiate PINK1 association to the OMM. MMP is decreased when CCCP is added which induces PINK1 association to the OMM. MMP and PINK1 are inversely related. This led to the prospect that MMP could be recovered after CCCP is removed to cause dissociation of PINK1 from the OMM.

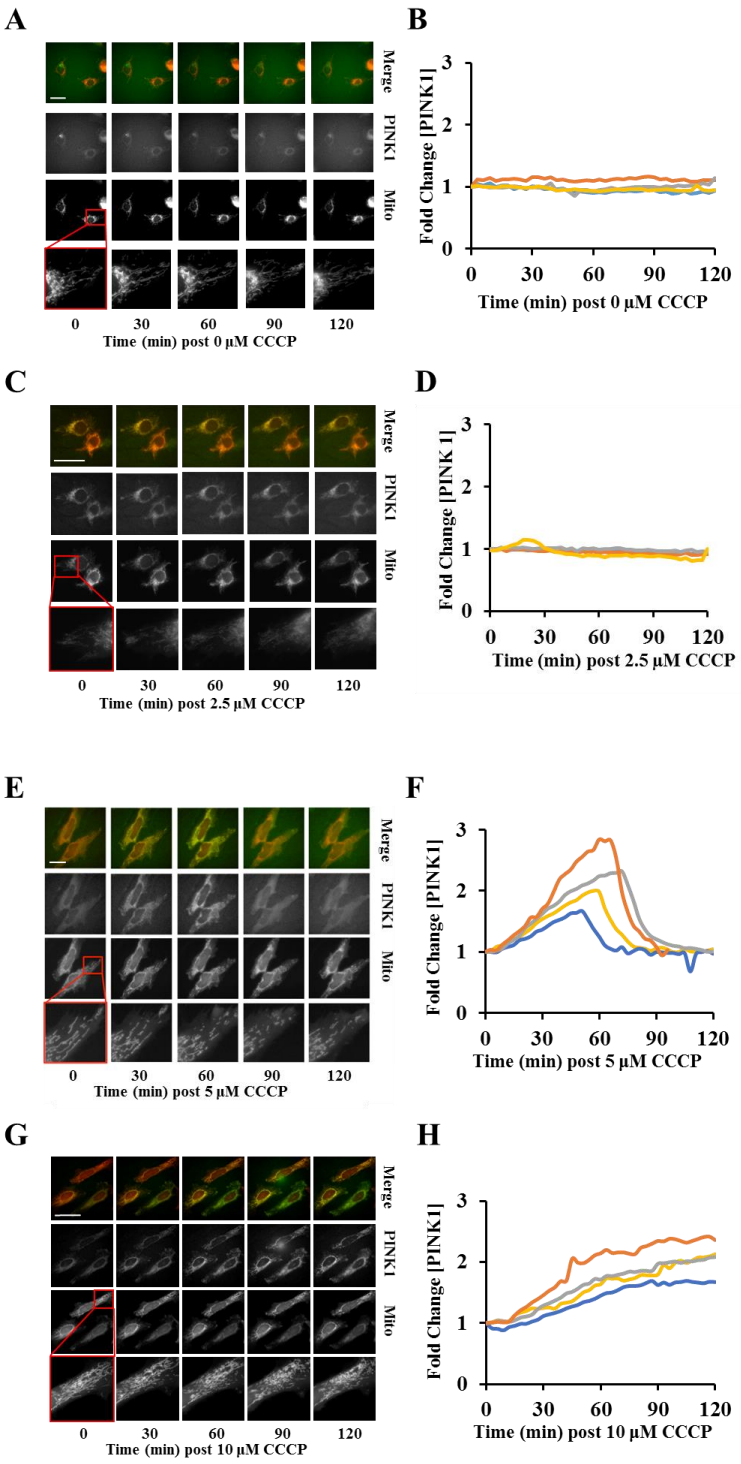

Figure 4. Modulation of PINK1 association and dissociation by titration of CCCP levels. A, C, E, and G. HeLa cells expressing PINK1-EGFP [green] and mito-mCherry [red] exposed to CCCP doses stated above. Scale bar represents 50 µm. **B, D, F, and H. Change in PINK1-EGFP fluorescence in** 4 representative cells per condition. Fluorescence levels were quantified using FIJI.

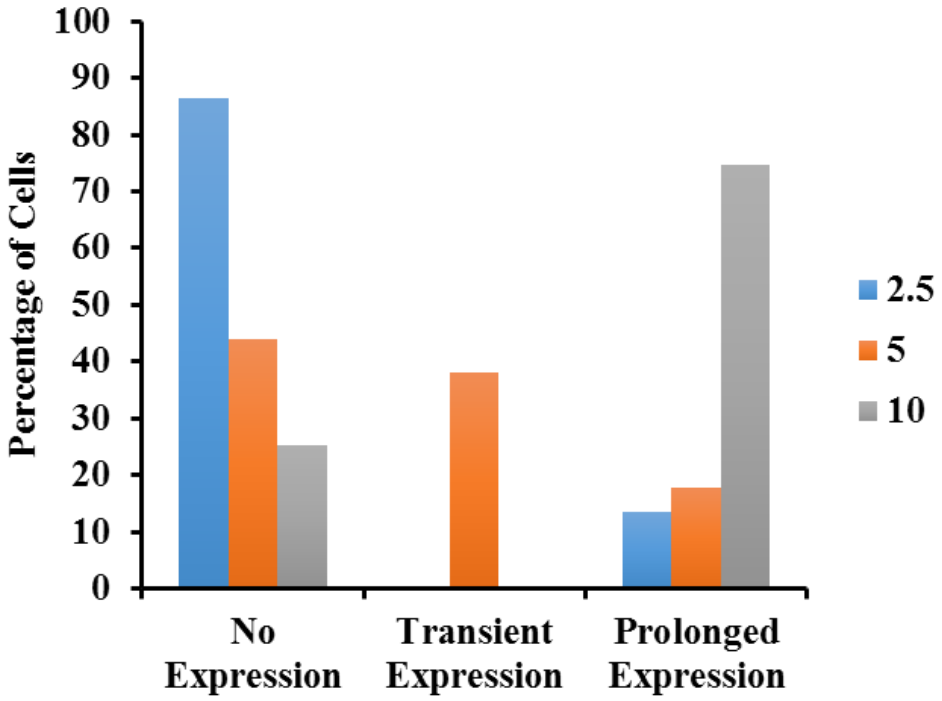

Figure 5. Semi-quantitative analysis of the PINK response in cells exposed to different CCCP doses. The kinetics of PINK1 response in cells exposed to different concentrations of CCCP for 2 h (as described in Fig. 4) were classified as either 'No expression', where no increase in PINK1-EGFP fluorescence was observed post-CCCP, 'Transient Expression', if PINK1-EGFP levels increased but returned to basal levels within the 2 h assay period, or 'Prolonged Expression' is PINK1-EGFP levels increased post CCCP and remained elevated at the end of the 2 h assay period.

Recovery of mitochondrial membrane potential post CCCP wash-out induces an increase in mitochondrial membrane potential.

To determine whether fluctuations in MMP at intermediate CCCP doses were the cause of the transient PINK1 response observed in cells dosed with 5 µM CCCP, we artificially generated large MMP fluctuations by transiently exposing cells to high doses of CCCP (10 µM) and then washing it out of the cells and later washing the cells and placing

CCCP media back onto the cells. This was possible as CCCP is a reversible inhibitor of mitochondrial oxidative phosphorylation. As before, MMP was measured by staining the cells with TMRM. To test the effects of 'pulsing' CCCP, CCCP was added initially and then removed and placed back on the cells 20 minutes later. All of the cells tested showed a slight peak of signal after the wash (Figure 6A, t = 20 min). By adding this high dose to cells, removing it, and adding it again, it was determined that 'pulsing' CCCP can also control MMP (Figure 6B). In this particular experiment, the artificial fluctuations in MMP caused by CCCP pulsing were large and it was unclear whether the naturally-occurring, smaller fluctuations in MMP observed in cells exposed continuously to intermediate doses of CCCP would cause transient PINK1 accumulation at the OMM.

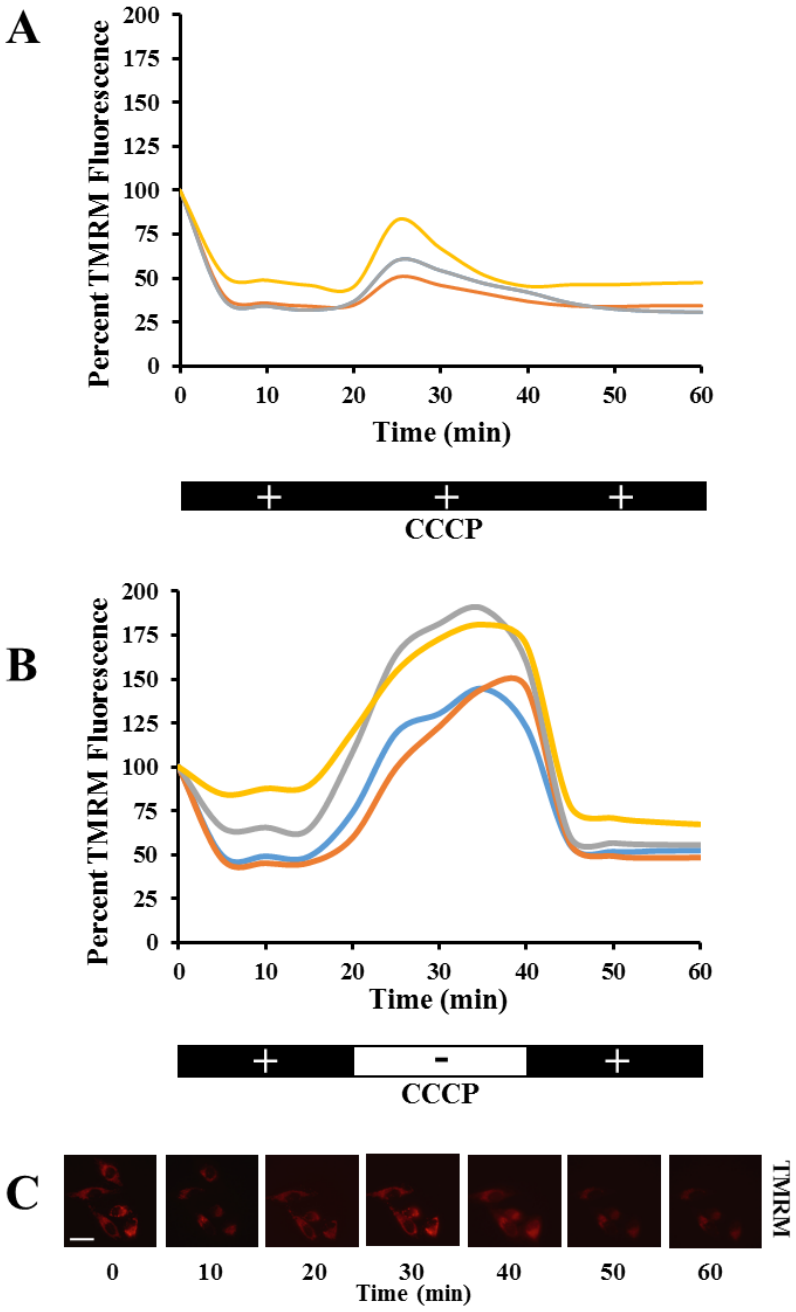

Figure 6. Mitochondrial membrane polarization is restored after CCCP washout.. A. TMRM-loaded HeLa were treated with 10 µM CCCP and imaged for 20 min. HeLa cells were washed and media containing TMRM and 10 µM CCCP was immediately added back. **B.** TMRM-loaded HeLa cells were treated with CCCP . At t = 20 min post CCCP, the CCCP was washed out and the cells were imaged in normal growth medium (without CCCP) for a further 20 min. These cells were then treated again with CCCP for the final 20 min of the imaging period. **C.** Timecourse images from the experiment described in **B**. Scale bar represents 50 µm.

Small fluctuations in mitochondrial membrane polarity induce rapid dissociation of PINK1 from the outer mitochondrial membrane

In order to validate the association between PINK1 fluorescence and mitochondrial membrane potential, PINK1-EGFP was expressed in cells treated with TMRM so that PINK1 levels and MMP could be monitored simultaneously in the same cells. We expected that dissociation of PINK1 from the OMM would be preceded by increases in MMP.

As predicted, HeLa cells treated with 5 μM CCCP doses displayed a decrease in MMP (as reported by a decrease in TMRM fluorescence) with a concomitant increase in PINK1 expression. In some cells, we subsequently observed a small and transient increase in MMP (starting around 75 min), and at the same time, PINK1 began to rapidly dissociate from the outer mitochondrial membrane (Figure 7A). These findings are represented graphically for four individual cells in Figure 7B. These data showed that PINK1 dissociation and MMP recovery were related. Importantly, these data also show that relatively small increase in MMP are sufficient to induce loss of OMM PINK1 and that complete recovery of basal MMP, as might have been expected, is not required.

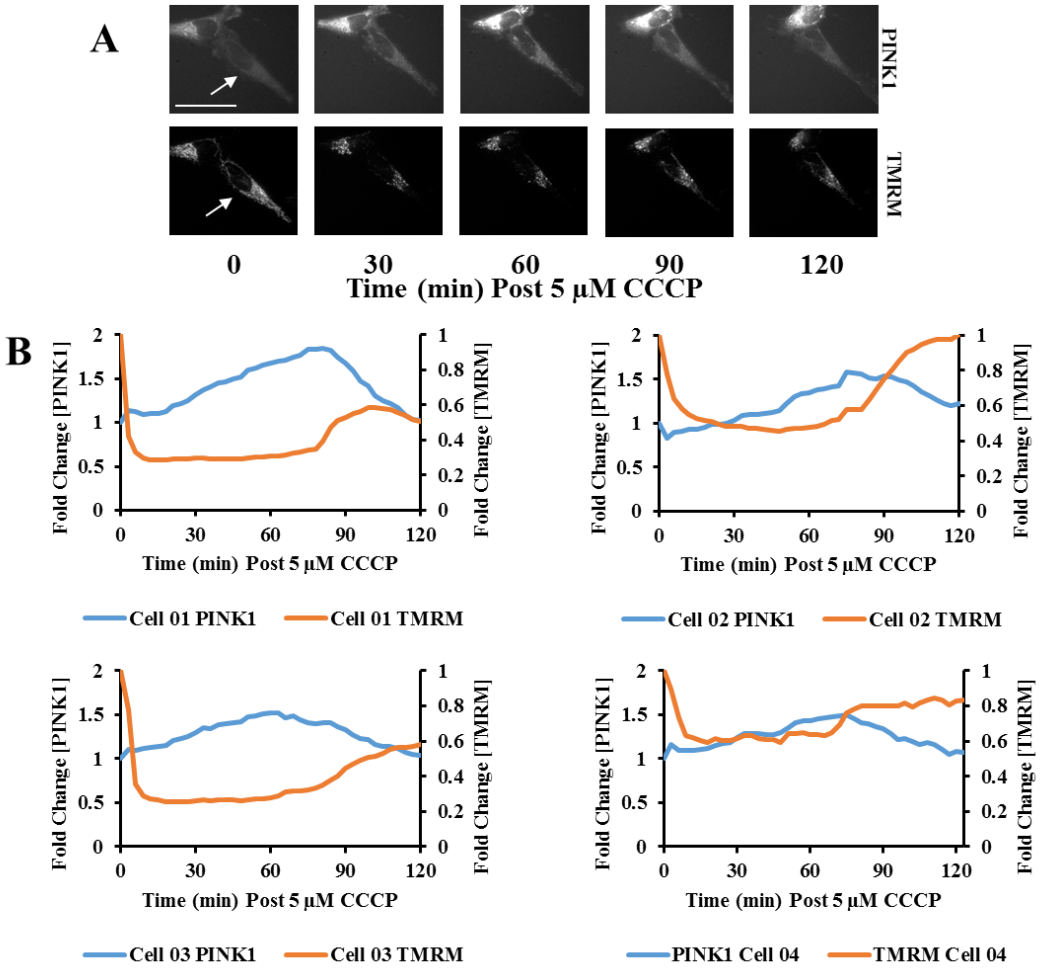

Figure 7. Small fluctuations in mitochondrial membrane potential result in dissociation of PINK1 from mitochondria. **A.** TMRM-loaded HeLa cells expressing PINK1-EGFP were treated with 5 µM CCCP and immediatedly imaged by fluorescence microscopy. Decreased TMRM fluorescence was concomitant with an increase in PINK1-EGFP fluorescence. Small increases or complete restoration of basal TMRM fluorescence was accompanied by a decrease in PINK1-EGFP fluorescence. **B.** Quantification of TMRM and PINK1-EGFP fluorescence in 4 representative cells. Cell 01 is represented in **A** (denoted by arrow).

Discussion

The process of mitophagy is absolutely essential for the maintenance of cellular health and viability. This is especially apparent in DA neurons of the SNpc. These extremely high energy dependent neurons extensively utilize mitophagy to maintain their vast mitochondrial networks (Chen and Chan 2009). Without the removal of damaged mitochondria through mitophagy, the mitochondrial network becomes compromised and which may result in the death of the cell. Impairment of mitophagy in DA neurons can result from autosomal recessive genes encoding mutations in PINK1 and Parkin, two proteins essential for mitochondrial autophagy (Nuytemans *et al*. 2010). In the mitophagy pathway, PINK1 is arguably of utmost importance surveilling and marking dysfunctional mitochondria for destruction, even without the presence of Parkin, albeit in a less efficient manner than PINK1:Parkin.

Although the basic components of the mitophagy pathway have been identified, exactly how they operate individually and in unison to correctly respond to different mitochondrial insults has yet to be fully resolved. For example, the importance of PINK1 has been explored through mitophagy studies that simulate catastrophic and complete depolarization of mitochondrial membrane potential. This may not closely model the stresses experienced by DA neurons in PD, which develops slowly over decades. However, recent studies have shown that the PINK1:Parkin pathway is capable of mediating different cellular fates in response to different mitochondrial poisons (Zhang *et al*. 2014). In this study we sought to further understand how PINK1 responds to different mitochondrial damage signals, specifically, mid to low range mitochondrial stresses. In so doing, we will

gain new insights into how persistent, low level mitochondrial stresses and the natural fluctuations in mitochondrial membrane potential observed in health cells are 'interpreted' and acted upon by the mitophagy system.

In order to effectively study PINK1, we developed a set of new reagents, pPINK1-mCherry and pPINK1-EGFP that allowed us to determine the levels and localization of PINK1 in live cells by fluorescence microscopy. Next, we successfully validated these constructs by gel electrophoresis and Western blotting (Figures 1 and 2), demonstrating that these fluorescent fusions of PINK1 could be expressed in mammalian cells and were stabilized in response to mitochondrial depolarization. Once the reagents were transfected into cells and tested, to our surprise we found that doses of 5 µM CCCP were found to induce transient expression of PINK1 on the OMM. While the kinetics of PINK1 stabilization were similar to that observed in cells exposed to maximal doses of CCCP (i.e. 10 µM), the dissociation of PINK1 from the mitochondria and its degradation was extremely rapid, occurring within ~15 min. Further testing revealed that these transient PINK1 responses were directly linked to MMP. Contrary to what might be expected, we observed that full repolarization of the mitochondria was not necessary to trigger complete loss of mitochondrial PINK1. Small increases in MMP appeared to be sufficient.

These transient responses indicated that PINK1 does not operate as a simple binary switch, producing an all-or-nothing response at a particular threshold MMP. Instead, our data indicates that PINK1 will associate with the OMM if significant, but not necessarily completed mitochondrial depolarization is experienced. However, should MMP show small signs of recovery, PINK1 initiates something similar to "self-destruct" function, quickly aborting the mitophagy process before mitochondria are unnecessarily destroyed.

We speculate that this mechanism is driven by the loading of PINK1 into the OMM during mitochondrial depolarization, allowing it to be rapidly 'flipped-in' to the intermembrane space within the mitochondria by MMP-dependent TIM:TOM transporters when mitochondrial function is partially restored where proteases cleave PINK1 releasing it back into the cytoplasm for proteasomal degradation. We suggest that by operating in this fashion mitochondria destruction in response to minor damage can be avoided, preventing the unwanted destruction of otherwise healthy mitochondria experiencing fluctuations in membrane potential.

Healthy cells experience fluctuations in MMP regularly. PINK1's ability to transiently associate to the OMM allows for cells to not hastily destroy undamaged mitochondria. In the case of neurons, 95% of their ATP demands are met by oxidative phosphorylation from their own mitochondria (as reviewed by Mironov 2009). If neurons destroyed their mitochondria after small fluctuations in MMP, then they would lose their primary source of ATP.

In the context of PD, neuronal death does not occur rapidly. The onset of PD lasts for many decades with a gradual buildup of dysfunctional DA neurons and a gradual increase in associated symptoms. Based off of the results collected in this study, we believe that transient, low levels of mitochondrial stress may be maintained over many years. This constant, low-grade stress on the mitochondria may damage the mitochondria by changing their morphology and decreasing their overall energy output. The mitophagy system does not seem to respond well to catching low levels of damage. Over time these low levels may result in the accumulation of dysfunctional mitochondria and cause the disruption of energy

production in the energy sensitive DA neurons. Thus, leading to their death and the formation of PD.

In order to explore this theory further, Parkin needs to be expressed in cells along with PINK1 to determine how the effects observed at the PINK1 level propagate to downstream components of the mitophagy pathway, and ultimately how it affects the mitochondrial mass of the cell. Transient levels of damage must also be stimulated in a DA neuronal cell line to understand neuronal PINK1 dynamics and their function in a more appropriate PD model. The HeLa carcinoma cell line has provided ample information, but to completely understand mitophagy in the context of PD, neurons are necessary.

This project, consisting of a completed and novel set of experiments designed to investigate the partial impairment of mitochondrial membrane potential concludes, that the responses of the PINK1 component of the mitophagy pathway have possible evolved to 'filter-out' inconsequential, transient fluctuations in MMP, thereby preventing the inappropriate loss of healthy or repairable mitochondria. The new insights elucidated in this thesis will aid in continued mitophagy study and may lead to the design of therapeutic strategies to prevent and treat PD.

Definition of Terms

Anosmia – loss of ability to smell

Apoptosis –programmed cell death.

Autophagy – cellular degradation of cytosolic components in the lysosome.

Bradykinesia – slow movement

CCCP - a depolarizing agent that diminishes mitochondrial membrane potential in selected cells by inducing H^+ permeability to the mitochondrial membrane

CDS – Coding sequence of a gene

Dementia – a decline in mental ability that affects daily life

Depolarize – reduction of a membrane resting potential

DNA ligase – an enzyme that catalyzes the formation of phosphodiester bonds in DNA

Dopaminergic neurons – neurons in the brain that produce dopamine, a neurotransmitter

E3 ubiquitin ligase – a protein that facilitates the binding of ubiquitin by recruiting a conjugating enzyme loaded with ubiquitin and aiding in its transfer to a protein substrate

Ethidium Bromide – intercalating agent used as a fluorescent dye for protein visualization in agarose gels

Fusion protein – chimeric proteins, joining of two or more genes that code for separate proteins

HeLa cells – an immortal cell line of cervical cancer cells taken from Henrietta Lacks. These cells are commonly used in research due to their toughness and prolific nature.

Hypomimia – a reduction in facial expression resulting from loss of motor control

Immunoblotting – utilization of antibodies to identify specific proteins from unrelated ones

Kanamycin – an aminoglycoside bacteriocidal antibiotic

Ligate – the linking of nucleotides in DNA and RNA with phosphodiester bonds

mCherry – a fluorescent protein with excitation wavelength of 575 nm and emission wavelength of 610 nm

Mitochondria – an organelle in cells that manages the processes of respiration and energy production

Mitofusins 1 and 2 – essential proteins for the maintenance of mitochondrial fusion and shape

Mitophagy – degradation of mitochondria through the process of autophagy, dysfunctional mitochondria are transported to lysosomes to be degraded

MitoTracker Deep Red FM – fluorescent dye used in live cells to visualize mitochondria localization

Parkin – an E3 ubiquitin ligase involved in the MQC pathway.

Parkinson's disease – neurodegenerative disease characterized by loss of DA neurons in the SNpc causing a decrease in motor control

PARL – presenilins-associated rhomboid-like protein, intramembrane serine protease responsible for the cleavage of PINK1 in healthy mitochondria

PCR – polymerase chain reaction, process used in molecular biology to amplify sequences of DNA

Phosphorylate – the introduction of a phosphate group to a molecule causing it to change to its active state

PINK1 – PTEN-induced putative kinase 1, a protein that accumulates on the surface of damaged mitochondria and signals for their degradation by recruiting and activating Parkin

Plasmid – circular DNA commonly found in bacterial cells that can replicate independently from chromosomal DNA

Primers – a strand of nucleic acids used as a starting point for DNA polymerase to begin DNA synthesis

Protease – any enzyme that catabolizes peptide bonds in the amino acid sequence of proteins

Proteasomal degradation – the breakdown of proteins by proteases through the hydrolysis of peptide bonds in the amino acid sequence of proteins

Restriction enzyme – enzymes that cut DNA at or near specific nucleotide sequences (e.g. *EcoR1* and *BamH1)*

Retropulsion – loss of balance in the posterior direction

SDS-PAGE – sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, SDS is a detergent used to unfold proteins to allow their separation by molecular weight on polyacrylamide gel

Substantia nigra pars compacta – a region of the brain involved with motor control that is rich in dopaminergic neurons

Transfect – the deliberate introduction of specific nucleic acid sequences to cells

Transformants – cells that have successfully taken up and integrated transfected DNA into their genetic sequence

Ubiquitination – a post-translational modification where ubiquitin is added

Western Blotting – a process by which specific proteins are detected in a sample of cell or tissue extract through the use of polyacrylamide gels and immunoblotting

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