Regulation and Activation of PARK2-mediated Mitophagy

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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2016

Abstract

An accumulation of damaged mitochondria by reactive oxygen species (ROS) can trigger apoptosis. This process has been implicated in neurodegenerative diseases such as Parkinson’s disease (PD). About 10% of the PD cases are caused by mutations in the PARK genes that are inheritable. The product of PARK2, Parkin, can mediate the autophagic degradation of damaged mitochondria. Autophagy is a process by which bulky cytoplasmic contents are degraded by lysosomes. PARK2 is recruited to the damaged mitochondria and ubiquitinates outer mitochondrial proteins. Ubiquitin serves as an important signal to recruit autophagy machineries to the mitochondria and deliver mitochondria to the lysosomes.

Even though the major molecular events in PARK2-mediated mitophagy are revealed, how this pathway is activated and regulated remains unclear. In my thesis, I identified two deubiquitinating enzymes, ubiquitin specific protease 30 (USP30) and 35 (USP35), that can regulate PARK2-mediated mitophagy. I showed that USP30 antagonizes PARK2 activity by deubiquitinating some of the PARK2 substrates, interfering with PARK2-substrate interactions, and delaying PARK2 recruitment to the mitochondria. USP35 affects the protein and mRNA
levels of mitofusins 2 as well as PARK2-substrate interactions. However, USP35 did not interfere with PARK2 recruitment. Furthermore, I described the development and characterization of a technique to activate mitophagy with a photo-sensitizer, mitochondrial-targeting KillerRed (mtKR). I showed that a sudden increase in ROS levels in the mitochondria induces mitochondrial depolarization and recruitment of PARK2. I also provided evidence that elongated mitochondria are more resistant to mtKR-induced mitophagy while fragmented mitochondria are more prone to mitophagy activation.
Acknowledgments

First of all, I want to thank my supervisors Dr. G. Angus McQuibban and Dr. Peter K. Kim for their guidance, advice, and support. You offered me a chance to work in one of the hottest fields in mitochondria research and provided me with a platform to develop and grow as a scientist. You allowed me to explore my own ideas and pushed me forward when I felt discouraged and lost. I will take your wisdom and knowledge with me anywhere I go. I would like to thank my committee members, Dr. John Brumell and Dr. David Williams for their endless help and support. Your unique insight, scientific advice, and constructive criticism helped my projects grow and flourish.

I would have not made it here without the generous support from McQuibban Lab and Kim Lab members: Jeff, Mod, Eliana, Guang, Riya, Tasha, Mauro, Rediet, Eliane, Natalia, Wendy, Liz, Yulia, Rong, Milu, Kelsey, Adriano, Derrick, Sasha, and Tatiana. You guys made my Ph.D. a fun and exciting experience. Special thanks to Mauro, Milu, Riya, Tasha, and Eliane for their hard work on the “DUB project”. We were a great team!

I am grateful for my parents who gave me the chance to live and study in Canada and supported my decision to go to graduate school. They always believe in me and my ability to succeed. Their love and guidance shaped me into the person that I am today, and I think that they have done a pretty good job.

Finally, I would like to thank Graeme. I am lucky to have met you in graduate school and worked with you side-by-side for the past five years. You are my colleague and my friend, who is always there for me through the good times and the bad ones. I am forever grateful for your love and support.
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Chapter 1

1 INTRODUCTION

1.1 Summary

Mitochondria are often viewed as a “double-edged sword” in the mammalian cells. On one hand, they provide the basic energy unit to a plethora of cellular processes that are essential for survival and growth. However, damaged mitochondria, if not properly repaired or eliminated, can release factors that trigger programmed cell death. In this introduction, I will first describe the dynamic properties of mitochondria and how mitochondrial damage occurs. Secondly, I will introduce the role of mitochondria in Parkinson’s disease. Next, I will introduce a pathway that cells utilize to degrade damaged mitochondria, termed mitophagy, and its implication in Parkinson’s disease. Lastly I will discuss how mitophagy is regulated and the significance of studying this process.

1.2 Mitochondria are one of the main productions sites of reactive oxygen species (ROS)

1.2.1 Mitochondrial dynamics

The dynamic properties of the mitochondria are achieved by several machineries that help them move, fuse, and divide. The mitochondrial fusion and fission machineries are highly conserved from yeast to human, suggesting the importance of these events (Westermann, 2010). Mitochondria has four distinct environments: outer-mitochondrial membrane (OMM), intermembrane space (IMS), inner-mitochondrial membrane (IMM), and matrix. When mitochondria fuse and divide, they need to ensure that all four environments are kept intact and distinct from each other. Mitochondria contain their own DNA, called mitochondrial DNA (mtDNA), which resides within the mitochondrial matrix and is essential for mitochondrial respiration. Unlike nuclear DNA, each cell contains many copies of mtDNA, from 100 to 10,000 depending on the cell type. mtDNA are susceptible to reactive oxygen species (ROS)-induced mutations and the accumulation of these mutations can be deleterious to the mitochondria.
Due to the lack of DNA repair mechanism in the mitochondrial matrix, it is important to ensure that all mitochondria have copies of undamaged mtDNA (Pamplona, 2011). In general, it is thought that mitochondrial fusion promotes content mixing and serves as a mechanism to repair damaged mitochondria. Conversely, when a portion of the mitochondria is too damaged, it needs to be isolated from the healthy mitochondrial network through the process of fission (Twig & Shirihai, 2011).

### 1.2.1.1 Mitochondrial Fusion Machinery

In mammals, the fusion machinery is composed of mitofusins 1 and 2 (MFN1/2) on the OMM and optic atrophy 1 (OPA1) on the IMM (Hoppins & Nunnari, 2009). Mammalian tissue culture cells lacking MFN1/2 or OPA1 exhibit fragmented mitochondria (Chen et al, 2003; Griparic et al, 2004). MFN1 and MFN2, which are paralogs sharing high sequence similarity with each other, have two transmembrane domains (TM), 2 coiled-coil domains, and a GTPase domain. The C-terminus, the N-terminus, and the functional domains of MFN1/2 are facing the cytosol (Zhao et al, 2013). It is believed that MFN1/2 from the adjacent mitochondria form dimers in the cytosol through interactions of the coiled-coil domains, MFN1/2 pulls the two opposite membranes close to each other and allows lipid mixing (Koshiba et al, 2004). Electron microscopy of a mitofusin homolog from cyanobacteria revealed that at GTP-bound state, mitofusin may promote membrane curvature and tabulation in order to promote lipid mixing from the opposite membranes (Low & Löwe, 2006).

Even though MFN1 and MFN2 can complement each other in the context of mitochondrial fusion, they may have different functions (Ishihara et al, 2004). Purified MFN1 have higher GTP hydrolysis activity than MFN2. Furthermore, MFN1 has a stronger tendency to form homo-dimers in the presence of GTP compared to MFN1-MFN2 hetero-dimers and MFN2 homo-dimers, suggesting that the initial tethering of mitochondria may depend on the activity of MFN1, not MFN2. Conversely, MFN2 is found concentrated at mitochondrial-endoplasmic reticulum (ER) contact sites. MFN2<sup>−/−</sup> cells exhibits phenotypes such as loss of mitochondrial-ER tethering and mitochondrial Ca<sup>2+</sup> uptake disruption, which can only be rescued by WT MFN2, but not MFN1 (de Brito & Scorrano, 2008).

OPA1 is a large GTPase protein responsible for IMM fusion. Rujiviphat et al. demonstrated that the short isoform of mitochondria genome maintenance 1 (s-Mgm1p), the
yeast orthologue of OPA1, promotes local membrane bending in vitro. Furthermore, the addition of GTP enhances the membrane bending property of s-Mgm1p. It is possible that by locally bending the IMM, s-Mgm1p or OPA1 can promote the fusion of adjacent membranes (Rujiviphat et al., 2015). In yeast, the OMM and IMM fusion events are thought to happen cooperatively because the OMM and IMM fusion machineries physically interact with a protein called Ugo1p (not found in mammals) (Hoppins & Nunnari, 2009). In mammalian cells, the fusion events of OMM and IMM are reported to happen in a sequential manner. The fusion of OMM is still facilitated by MFN1/2 in OPA1 null cells. Interestingly, EM tomography shows that OPA1 null cells have disrupted cristae structures, suggesting that OPA1 may play an important role in cristae maintenance in mammalian cells (Song et al., 2009).

1.2.1.2 Mitochondrial fission machinery

Mitochondrial fission is carried out by a large GTPase, Dynamin-related protein 1 (DNM1L). DNM1L facilitates mitochondria fission by forming a “ring” around the mitochondrial fission site. The DNM1L ring constricts around the mitochondria, causing the separation of mitochondrial content and membranes. The majority of the DNM1L is localized in the cytosol, only 3% of DNM1L can be seen on mitochondria under basal conditions (Smirnova et al., 2001). DNM1L does not have a mitochondrial targeting sequence (MTS), so it needs to be recruited to the mitochondria when needed. To date, there are 4 OMM proteins that recruit DNM1L to the mitochondria, FIS1, mitochondrial fission factor (MFF), and mitochondrial elongation factor 1/2. Knocking down any mitochondrial fission factor will lead to an inter-connected and elongated mitochondrial network (Losón et al., 2013). Little is known about how mitochondria fission sites are decided. Recent data suggest that ER play a crucial role in deciding where to divide a mitochondrion. Using electron microscopy and topography, Friedman et al. demonstrated that the ER wraps around mitochondria to indicate the mitochondrial constriction site in yeast and in mammalian cells. Moreover, the authors found that after ER wraps around the mitochondria, MFF and DNM1L are recruited to the ER-mitochondria contact site to mediate mitochondrial fission (Friedman et al., 2011).
1.2.2 Mitochondrial metabolism

Most mammalian cells rely on mitochondria for energy. The electron transport chain (ETC) residing on the IMM is responsible for most of the ATP production. The ETC couples the transferring of electrons with the generation of a proton gradient across the IMM. The ETC is composed of complex I, II, III, IV, and complex V (also known as ATP synthase). Complex I (CI) and complex II (CII) receive electrons from energized substrates, NADH and FADH$_2$, and pass the electrons to coenzyme Q10 (CoQ). NADH and FADH$_2$ harvest electrons from the oxidation of carbon sources, including pyruvate, fatty acids, and amino acids (Koopman et al., 2010). CoQ passes the electrons to the Q$_0$ site in complex III (CIII) (Rigoulet et al., 2011). CIII passes the electrons to complex IV (CIV) through cytochrome c, and the electrons are transferred to oxygen molecules to generate water in complex IV. CI, CIII, and CIV pump protons from the matrix to the IMS (Fig. 1.1) to generate the proton gradient. This proton gradient is utilized by the ATP synthase to generate ATP through the process of oxidative phosphorylation (Koopman et al., 2010). Due to the energy spent at the neuronal junctions, neurons have a particularly high demand on mitochondrial activity to generate ATP (Schwarz, 2013). Theoretically, one glucose molecule should yield 36 ATP molecules (Rich, 2003). However, because of the inefficiency in the ETC and the leakiness of the IMM (Mantel et al., 2011; Rabinowitz & White, 2010), fewer ATPs were produced in cells.

1.2.3 ROS production

The inefficiency in transferring electrons in CI and CIII results in the premature release of electrons. Superoxide generated by CI and CIII is considered an unavoidable by-product of ATP synthesis. In CI, superoxide can be produced by subunit NDUFV1 (NADH binding site) when there is a build-up of the electron donor, NADH (Koopman et al., 2010). It has also been reported that superoxide can be produced at the CoQ binding site when there is a build-up of reduced CoQ (Murphy, 2009). However, an accumulation of CoQ only happens during the reversed electron transport, and it is still unclear whether this is the dominant driver in CI superoxide production (Moreno-Sánchez et al., 2013). Interestingly, CI only releases superoxide into the mitochondrial matrix (Grivennikova & Vinogradov, 2006). In contrast, the semiquinone found in
the CIII Q₈ site may be exposed to and react with oxygen to produce superoxide and release it into the matrix and the IMS (Fig. 1.2) (Muller et al, 2004).

**Figure 1.1. The Schematic Representation of the Electron Transport Chain (ETC) in mammalian mitochondria.**

In the mitochondrial matrix, the tricarboxylic acid (TCA) cycle harvests energy from acetyl CoA and reduces NAD⁺ to NADH. One of the products in the TCA cycle, succinate, along with NADH serve as the energized substrates and donate electrons to complex I (CI) and complex II (CII) within the ETC. CI passes on the electron to complex III (CIII) through coenzyme Q10 (CoQ). In parallel to CI, CII also donates electrons to CoQ, which passes electrons to CIII. CIII passes electrons to complex IV (CIV) through cytochrome c (C in the schematic). Oxygen is the final recipient of the electrons and generates water. CI, CIII, and CIV pump protons across the inner-mitochondrial membrane (IMM) to generate the proton gradient. ATP synthase, also known as complex V (CV), allows protons to flow back into the matrix and uses the proton motive force to drive oxidative phosphorylation, which leads to the generation of ATP.
Figure 1. CI and CIII are the major contributor to superoxide production in the mitochondria.

CI generates superoxide in the NDUFV1 subunit, which is the NADH binding site. When there is an accumulation of NADH, oxygen molecules have access to the reactive site in CI and become electron recipients to generate superoxide. Due to the structure of CI, superoxide produced is only released into the mitochondrial matrix. CIII produces superoxide at the Q₀ site in the presence of an intermediate semiquinone radical. Even though the Q₀ site is close to the IMS, superoxide produced can be released to both the matrix and the IMS.

Superoxide is highly reactive and membrane impermeable (Rosen & Freeman, 1984). Therefore, superoxide produced in the mitochondrial matrix and IMS cannot escape the membrane barrier. Mitochondria contain Mn superoxide dismutase (SOD2) that quickly converts superoxide within the matrix into hydrogen peroxide (H₂O₂), a membrane permeable-form of ROS. Cu, Zn superoxide dismutase (SOD1) neutralizes the superoxide within the IMS (Kawamata & Manfredi, 2008). The neutralization of superoxide by SOD2 within the mitochondria is essential. Mitochondria isolated from SOD2−/− mice liver exhibited lower levels of oxygen consumption and more oxidative modification in mitochondrial proteins and mtDNA, implying that oxidative damage affects mitochondrial activity (Williams et al, 1998). H₂O₂ is further converted into water by glutathione peroxidase in the mitochondria or in the cytosol. Although more stable than superoxide, H₂O₂ can generate hydroxyl radical through the Fenton reaction (Orrenius, 2007). Hydroxyl radicals are the most reactive molecules among all ROS in the biological context (Halliwell, 1992) (Fig. 1.3). Notably, at low basal concentrations, ROS
serve as important signaling molecules involved in important cellular processes such as autophagy (Huang et al, 2011).

**Figure 1.3. Breakdown of superoxide**

Superoxide produced in the mitochondrial matrix is membrane impermeable and highly reactive. Superoxide is quickly converted to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase 2 (SOD2). H$_2$O$_2$ has the ability to cross membranes and is converted to water by glutathione peroxidase. H$_2$O$_2$ can also be converted to hydroxyl radicals (OH·), which are also highly reactive with biological molecules, such as DNA, lipid, and protein.

### 1.2.4 Oxidative damage of the mitochondria

The reactive nature of ROS means that they can damage macromolecules in their surroundings, including DNA, protein, and lipid. ROS can damage the sugar backbones, modify purines and pyrimidines, and create double-stranded and single-stranded breaks in mtDNA. In DNA, one of the oxidative modification products is 8-oxo7,8-dihydro-2’-deoxyguanosine (8-oxodG), which is commonly used as a marker to measure oxidative damage of the DNA. There is more 8-oxodG in mtDNA in comparison to nuclear DNA, possibly due to the proximity of mtDNA to the site of ROS generation (Pamplona, 2011). The DNA damages accumulated in the mitochondria are implicated in aging and neurodegenerative diseases such as Huntington’s disease (HD). HD is caused by mutations in Huntingtin gene, resulting in the expansion of the N-terminal glutamine repeats in the protein product. Siddiqui et al. showed that there was a significant decrease in mtDNA abundance and a higher frequency of mtDNA lesions in HD patients’ brains. In addition, cells containing mutant Huntingtin showed lower spare respiratory capacity compared to WT cells. The author hypothesized that the accumulation of mtDNA mutations or the deletion of
mtDNA reduce mitochondrial respiratory capacity, which further increases mitochondrial ROS production; therefore completing a vicious cycle of neuronal damage (Siddiqui et al., 2012).

At the protein level, ROS can modify several amino acid side chains, such as cysteine and tryptophan, resulting in a reduction in protein activity. Specifically, Graziewicz et al. demonstrated that mtDNA polymerase (pol γ) is inhibited by H₂O₂ in vitro and in vivo. When purified pol γ was incubated with exogenous 250 μM H₂O₂ for 1 hr, pol γ lost 50% of DNA polymerase activity. Furthermore, treating pol γ with increasing concentrations of H₂O₂ leads to a decrease in the pol γ’s ability to bind double stranded DNA (Graziewicz et al., 2002). It has been hypothesized that pol γ may perform repairs on mtDNA. Damaged pol γ may lead to accumulation of mtDNA mutation and exacerbate the problems caused by the mtDNA mutations.

Mitochondrial membrane is rich in unsaturated fatty acids and a prime target of mitochondrial ROS. Cardiolipin, an IMM lipid essential for mitochondrial bioenergetics, contains large amounts of unsaturated fatty acids (Paradies et al., 2011). Cardiolipin associates with cytochrome c and anchors cytochrome c to the IMM. This association can be disrupted when cardiolipin is oxidized, which creates a pool of soluble cytochrome c to be released into the cytosol in order to trigger apoptosis (Ott et al., 2002). Oxidized cardiolipin can sensitize mitochondria to Ca²⁺-induced depolarization, and has been implicated in neurodegenerative diseases and non-alcoholic fatty liver disease (Paradies, 2014). Taken together, without proper ROS management, mitochondria face great risk from oxidative damage, which can cause mitochondrial depolarization and trigger apoptosis.

1.2.5 Techniques that simulate ROS production in mitochondria

In order to study the outcomes of mitochondrial oxidative damage, one needs to either introduce ROS exogenously or induce ROS production in the mitochondria in mammalian tissue culture systems. Conventionally, the most direct method to stimulate oxidative damage is to incubate tissue culture cells with exogenous H₂O₂. H₂O₂ has the ability to enter cells via both diffusion across the membranes and through the aquaporins (Bienert & Chaumont, 2014). Singh et al. reported that treating HeLa cells with 125 μM H₂O₂ causes cytochrome c release from the
mitochondria, eventually leading to apoptosis activation in the cells (Singh et al., 2007). Low levels of \( \text{H}_2\text{O}_2 \) has been shown to induce mtDNA damage and to cause decrease in mtDNA copy numbers in prostate epithelial cells (Han & Chen, 2013).

While \( \text{H}_2\text{O}_2 \) provides a convenient method to induce oxidative stress in the mitochondria, the direction of ROS travelling through the cell is reversed. Under normal conditions, it is expected that ROS is generated in the mitochondria and diffused into the cytosol. An alternative method to induce oxidative damage is by generating ROS within the mitochondria. First discovered as a pesticide, rotenone is an inhibitor that prevents the electron flow from the iron-sulfur centre to the ubiquinone in CI (Grivennikova & Vinogradov, 2006). Treating mitochondria with rotenone results in an increase in superoxide production. Utilizing hydroethidium dye and flow cytometry, Li et al. showed that human promyelocytic leukemia cells produce more superoxide when treated with rotenone (Li et al., 2003). In 2011, Tanner et al. conducted a comprehensive study tying rotenone exposure to the development of Parkinson’s disease (PD), which makes rotenone treatment a biologically and pathologically relevant method to induce mitochondrial oxidative damage (Tanner et al., 2011). Rat phenochromocytoma and neuronal cells treated with 0.1-1 µM rotenone exhibit an increase in ROS production and a reduction in cell viability. Rotenone-induced ROS production leads to the activation of apoptosis, indicated by the increase in caspase-3 cleavage. When exogenous antioxidant enzymes were pre-incubated with the cells, there was a dramatic reduction in rotenone-induced ROS production and apoptosis activation. These results suggest that inhibition of CI activity leads to increase in ROS production and oxidative damage to the cells, and eventually results in apoptosis (Zhou et al., 2015).

\( \text{H}_2\text{O}_2 \) and rotenone treatments have provided us with knowledge on the cellular response to mitochondrial oxidative stress. However, these treatments induce global damage to the mitochondrial network, which may not be the ROS insult that cells face under physiological conditions. Recently, Ma et al. reported the observation of a sudden burst of superoxide in small regions of the mitochondria in the rat cardiac myocyte. These bursts of superoxide peak in ~3.5 s and disappear with the half time of ~8.6 s. The authors termed this phenomenon “superoxide flash” (Ma et al., 2011). Superoxide flashes were observed to coincide with transient depolarization in the same region of the mitochondria, suggesting a link between mitochondrial permeability transition pore activity and superoxide flashes. Ma et al. also reported that treating
cells with selenite induces superoxide flashes that precede apoptosis. Therefore, superoxide flash is thought to be an early indicator of ROS-induced apoptosis mediated by selenite treatment.

1.2.6 Mitochondrial-targeting KillerRed (mtKR)

Some fluorescent proteins, called photosensitizers, produce ROS upon excitation. Photosensitizers can be tagged in tandem with target proteins to render the target proteins inactive through oxidative damage when photosensitizers are photoactivated. In 2006, Bulina et al. generated a GFP homolog that produces almost 1000 fold greater amount of ROS upon photoactivation compared to GFP, which makes it an ideal photosensitizer. KillerRed (KR) is a fluorescent protein with an excitation maximum at 585 nm and an emission maximum at 610 nm. Expressing and photoactivating KR in E. coli can kill up to 96% of the cells. The authors also attached KR to the PH domain, which is a domain that interacts with the inner leaflet of the plasma membrane. Photoactivating KR reduced the affinity of the PH domain to phospholipid, suggesting that ROS emitted by KR can modify proteins in proximity (Bulina et al., 2006).

Analysis of the KR structure by x-ray crystallography revealed that it has a typical fold of the GFP family, a beta-barrel consisting of 11 sheets (Fig. 1.4). However, what makes KR distinctive from the other members of the GFP family is a channel within the beta-barrel that is filled with water molecules. This channel allows water molecules to have direct access to the chromophore centre of KR (Pletnev et al., 2009). It was proposed that the highly structured water channel may help electrons escape from the chromophore centre and react with oxygen molecules to generate superoxide. Conversely, it is also possible that the water channel helps oxygen diffuse to the chromophore centre. Easy access to the KR chromophore centre by oxygen may explain why KR is quickly photobleached during photoactivation (Roy et al., 2010). However, the precise mechanism of how KR produces superoxide remains unclear.

There are several potential advantages of utilizing KR to simulate mitochondrial oxidative damage. First, KR can be genetically encoded and expressed in tissue cultured cells. This allows one to target KR to specific subcellular locations within the cell. By attaching 2 MTS to the N-terminus of KR (mitochondrial-targeting KR or mtKR), Bulina et al. could localize mtKR to the mitochondria with relatively high levels of specificity (Bulina et al., 2006).
The second advantage of KR is that ROS production can be regulated. KR produces little ROS without photoactivation, hence the timing and the location of ROS production can be controlled by the precise laser activation of KR. Third, KR produces superoxide, which is the same ROS that CI and CIII produce to induce oxidative damage to the mitochondria. By targeting and photoactivating KR in the mitochondrial matrix, one can simulate a sudden elevation of superoxide within the mitochondria and study the outcome of this oxidative damage.

Figure 1.4. Crystal structure of KillerRed (KR).

(A) The crystal structure revealed that KR adopts a beta-barrel structure similar to that of the green fluorescent protein with the chromophore in the centre of the barrel. KR naturally exists in dimer. Source: adapted from Pletnev et al. 2009. (B) KR has a feature that is unique among GFP family members. It has a long water-filled channel in the centre of the barrel (shown in blue), which allows protons to directly access the chromophore centre (shown in red). There is also a bifurcated pore on the side of the barrel that also has access to the chromophore centre (shown in orange). Source: adapted from Roy et al. 2010.
1.3 Mitochondria and Parkinson’s disease

1.3.1 Parkinson’s Disease

Parkinson’s disease (PD) is a neurodegenerative disease that affects 1% of the population over the age of 55 worldwide (Navarro & Boveris, 2009). The onset of PD is associated with the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) located in the midbrain (Pogson et al., 2011; Damier et al., 1999). Dopaminergic neuron death results in a drastic reduction in the dopamine level in the brain (Gibb, 1997; Zarow et al., 2016). Dopamine is a neurotransmitter that is intimately involved in the motor control pathways (Vitrac et al., 2014), hence PD patients often exhibit symptoms such as muscle rigidity, resting tremors, and slowness of movement (bradykinesia) (Lin & Beal, 2006).

PD can be categorized into two types: familial PD and sporadic PD. I will be discussing familial PD in detail in sub-chapter 1.3.2. Sporadic PD (cases in which patients do not inherent genes that cause familial PD) accounts for 90% of all PD cases (Belin & Westerlund, 2008). Many environmental and internal factors can contribute to the development of sporadic PD; however, there is no single factor responsible. For example, as I previously discussed, the exposure to rotenone as a pesticide has been associated with increased PD cases among farmers in Iowa and North Carolina, yet not all farmers exposed to rotenone develop PD later in life (Tanner et al., 2011).

There are several hallmarks of PD. The first one is aging, which is a well-accepted risk factor. The chances of developing PD positively correlates with age (Hindle, 2010). Secondly, the presence of Lewy bodies in the SNpc is considered a major hallmark in PD (Beyer et al., 2009). A Lewy body is composed mainly of α-synuclein (SNCA) and other proteins forming an aggregate. The oligomerization and aggregation of SNCA is considered as the event preceding Lewy body formation. Once SNCA aggregate is formed, other proteins, such as SNCA-binding proteins and ubiquitin-protease proteins, will also aggregate with SNCA (Wakabayashi et al., 2007). The presence of Lewy bodies in areas that exhibit neuronal loss implies that Lewy bodies may be the cause of neuronal death (Tompkins & Hill, 1997). However, other evidence suggests that dysfunctional SNCA may be more toxic to neurons than the Lewy body itself, hence Lewy bodies may have protective functions to neurons (Tanaka et al., 2004). Another hallmark in PD is the oxidative stress of the neurons. Dopaminergic neurons are especially prone to oxidative stress
because of dopamine oxidation. Under normal conditions, dopamine is stored inside the synaptic vesicles in the neurons. These vesicles release dopamine into the synapses upon stimulation. However, factors such as SNCA fibril build-up can disrupt proper storage of dopamine in the cell (Hastings, 2009). Dopamine in the cytosol can be oxidized in the presence of oxygen molecules to form dopamine-o-quinone (Graham, 1978). Dopamine can also be converted to dihydrophenylacetic acid and H$_2$O$_2$ by monoamine oxidase (Maker et al, 1981). Oxidative stress of the neurons and the dopamine oxidation can create a vicious cycle that contributes to the death of dopaminergic neurons.

1.3.2 The role of mitochondria in PD

Mitochondria have long been suspected to be involved in PD. Several groups reported that CI activity is decreased in the brain of PD patients (Schapira et al, 1988; Janetzky et al, 1994). Moreover, Navarro et al. demonstrated that mitochondrial oxygen uptake and electron transfer activities in CI, CIII, and CIV were significantly reduced in PD patients’ frontal cortex, suggesting a role of mitochondrial quality in PD (Navarro et al, 2009). Perhaps, the most convincing evidence demonstrating that mitochondria quality control is tightly associated with the onset of PD is the discovery of genes related to familial PD (Table 1).

In 1997, α-synuclein (SNCA or PARK1) became the first gene identified in familial PD. Polymeropoulos et al. discovered two mutations in SCNA, A53T and G209A that cause an autosomal dominant form of familial PD (Polymeropoulos et al, 1997). In addition, duplication or triplication of the SCNA gene leads to familial PD, indicating an intrinsic toxic property of SCNA (Chartier-Harlin et al, 2004). Later reports showed that expressing mutant forms of SCNA cause mitochondrial depolarization, increase in cellular ROS, and activation of cytochrome c-mediated apoptosis, suggesting that mutant SCNA contributes to mitochondrial damage and cytotoxicity in PD (Choubey et al, 2011). Curiously, overexpressing WT SCNA in mammalian cells causes mitochondrial fragmentation that is independent of mitochondrial fusion or fission machineries (Kamp et al, 2010). SCNA has lipid-binding properties and interrupts membrane fusions in vitro, therefore high SCNA levels may disrupt mitochondrial dynamics and cause mitochondrial damage. SCNA can also form oligomeric structures in vitro, known as protofibrils. Structural analysis revealed that mutant SCNA$^{A53T}$ and SCNA$^{A30P}$ promote protofibril
formation in vitro. Moreover, electron microscopy analysis showed that protofibrils form a pore-like structure, implying that the SCNA protofibrils may form pores on the mitochondrial membrane and disrupt mitochondrial functions (Lashuel et al, 2002).

*DJ-1 (PARK7)* is another gene that associates mitochondrial quality control with PD. Deletion of the *DJ-1* gene and point mutations were identified in Dutch and Italian PD patients (Bonifati et al, 2003). DJ-1 has antioxidant activities. Purified DJ-1 can eliminate 80% of H₂O₂ when incubated together in vitro. Overexpressing WT *DJ-1* protects neuroblastoma cells from apoptosis induced by H₂O₂ treatment (Taira et al, 2004). *DJ-1*⁻/⁻ neurons have fragmented mitochondria and are more susceptible to cell death induced by MPTP (a Parkinsonism-inducing drug) treatment. *DJ-1*⁻/⁻ neurons showed increased production of H₂O₂, primarily through the inhibition of complex I activity. Moreover, treating *DJ-1*⁻/⁻ neurons with an antioxidant, N-acetyl-L-cysteine (NAC) rescues the mitochondrial fragmentation phenotype (Irrcher et al, 2010). These data hint at a connection between mitochondrial dynamics and DJ-1 antioxidant functions.

SNCA and DJ-1 mutations constitute to a small percentage of familial PD cases. However, PARK2 and PTEN-induced kinase 1 (PINK1 or PARK6) together account for 51% to 57% of familial PD (Pankratz & Foroud, 2007). Subsequent studies showed that PARK2 and PINK1 are involved in the quality control of mitochondria via a process called selective autophagy.
Table 1. Genetic Causes of Parkinson’s Disease.

<table>
<thead>
<tr>
<th>PARK loci</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Form of PD</th>
<th>Mutations</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1</td>
<td>SNCA</td>
<td>4q21</td>
<td>AD</td>
<td>A30P, E46K, A53T</td>
<td>Greece and Italy</td>
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<tr>
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<td>Parkin</td>
<td>6q25.2–q27</td>
<td>AR J</td>
<td>Various mutations, exonic deletions, duplications and triplication</td>
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<tr>
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<td>2p13</td>
<td>AD</td>
<td>–</td>
<td>Europe</td>
</tr>
<tr>
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<td>4q21</td>
<td>AD</td>
<td>Duplication and triplication</td>
<td>Iowa</td>
</tr>
<tr>
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<td>UCHL1</td>
<td>4p14</td>
<td>AD and idiopathic</td>
<td>I93M and S18Y</td>
<td>Germany</td>
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<tr>
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<td>G309D, exonic deletions</td>
<td>Italy</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>1p36</td>
<td>AR and EO</td>
<td>Homozygous exon, deletion L166P</td>
<td>Europe</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>12q12</td>
<td>AD and idiopathic</td>
<td>R1441C/G/H, Y1699C, G2019S, I2020T, G2385R</td>
<td>Japan</td>
</tr>
<tr>
<td>PARK9</td>
<td>ATP13A2</td>
<td>1p36</td>
<td>Kufor–Rakeb syndrome and EO PD</td>
<td>Loss-of-function mutations</td>
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<td>–</td>
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<td>AD and idiopathic</td>
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<td>North America</td>
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<td>Familial</td>
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<td>North America</td>
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<tr>
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<td>HTRA2</td>
<td>2p13</td>
<td>Idiopathic</td>
<td>A141S, G399S</td>
<td>Germany</td>
</tr>
</tbody>
</table>

AD, autosomal dominant; AR, autosomal recessive; EO, early-onset; J, juvenile. Source: Taken from Belin and Westerlund, 200
1.4 PINK1/PARK-mediated mitophagy

1.4.1 Autophagy

Ashford and Porter in 1962 observed that there was a sharp increase in lysosome numbers after treating rat liver with glucagon for 4 hrs. They found traces of mitochondria and other cytosolic components within these lysosomes. Christian de Duve, famously coined the term, “autophagy” (“self-eating” in Greek) to describe this process (Yang & Klionsky, 2010b). It is a cellular process in which double-membrane organelles grow around and engulf cytosolic content, and fuse with lysosomes in order to degrade the content (Xie & Klionsky, 2007). Besides glucagon treatment, amino acid deprivation is another trigger for autophagy activation in rat liver (Mortimore & Schworer, 1977). The major purpose of autophagy is to recycle essential nutrients during starvation and to remove damaged organelles or invading pathogens. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Gomes & Scorrano, 2012). I will be only discussing macroautophagy (herein referred as autophagy) in my thesis. Our understanding of the autophagy pathway comes from yeast genetic screens (Ohsumi, 2006). Thirty-one genes were identified to facilitate the autophagy pathway in yeast, these were later renamed as ATG genes (Inoue & Klionsky, 2010). To date, a total of 37 ATG genes are found in yeast (Nazarko et al, 2014).

Mammalian cells have similar core complexes to facilitate autophagy as yeast. In the mammalian system, ULK1-ATG13-FIP200 complex (Atg1 complex in yeast) is responsible for autophagy induction (Yang & Klionsky, 2010a). ULK1 requires ATG13 and FIP200 to localize to the isolation membranes and to become activated (Ganley et al, 2009). Phosphatidylinositol 3 kinase complex (BECN1-ATG14-VPS34) induces vesicle nucleation to form a double-membrane structure called a phagophore (Burman & Ktistakis, 2010). Through two ubiquitin-like conjugation systems, microtubule associated protein 1 light chain (LC3) (or Atg8p in yeast) is cleaved into mature form, lipidated, and inserted onto the phagophore membrane, which helps the expansion and the formation of autophagosomes. There are seven mammalian homologues of Atg8p, LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GATE16, and ATGL8, among which LC3B (referred to as LC3 in this thesis) was studied most extensively (Tanida et al, 2004). Phagophores engulf cytosolic components or damaged organelles to form autophagosomes. Facilitated by SNARE proteins, VAMP8 and Vtilb, matured autophagosomes fuse with the
lysosomes to form an autophagolysosome (Furuta & Amano, 2010). Lysosomal hydrolases degrade the content within the autophagolysosomes and release essential nutrients back into the cytosol (Yang & Klionsky, 2010a) (Fig. 1.5).

**Figure 1.5. The schematic representation of the macroautophagy pathway.**

Unc-51 Like Autophagy Activation Kinase 1 (ULK1) complex and the Beclin-1 (BECN1) complex are responsible for autophagy activation and membrane nucleation respectively. Together, these two complexes initiate the formation of the phagophore, a double membrane structure that grows around the cytosolic content designated for degradation. Through two ubiquitin-like conjugation systems, LC3 is lipidated and incorporated onto the phagophore membrane to drive the growth of membrane to form an autophagosome. Autophagosomes fuse
with lysosomes to form autophagolysosomes, in which lysosomal hydrolases degrade the content.

1.4.2 Role of ROS in autophagy

Despite the traditional views of ROS as a harmful molecule, current research has shed light on the functional aspects of ROS, suggesting that they participate in various signaling events. ROS has been shown to regulate cell proliferation, immune response, and autophagy. Because ROS can modify many members in the autophagy pathway, they play a complex role in autophagy regulation (Huang et al., 2011). Utilizing ROS dyes that are specific for superoxide or \( \text{H}_2\text{O}_2 \), Chen et al. demonstrated that superoxide is the major ROS that regulates autophagy in starved cells. Moreover, overexpression of SOD2 reduced starvation-induced autophagy, demonstrated by the reduction in LC3 processing (Chen et al., 2009). However, the target of ROS in the autophagy pathways remained unclear in this report.

ROS has been shown to activate non-canonical autophagy pathways. Wong et al. reported the discovery of a small molecule, 1,3-dibutyl-2-thiooxo-imidazolidine-4,5-dione (C1), that specifically induces cell death in cancer cells, but not in benign cell lines. While uncovering the mechanism of C1-induced cell death, the authors noticed that C1 induces LC3 labelled puncta formation in these cancer cell lines. Surprisingly, C1 activates autophagy in BECN1 and ULK1 depleted cells, suggesting that it activates a non-canonical autophagy pathway. Cells treated with C1 showed increase \( \text{H}_2\text{O}_2 \) and superoxide levels, which led to the activation of the ERK/JNK pathway. The authors hypothesized that ERK/JNK may be able to induce autophagy in a BECN1-independent manner (Wong et al., 2010).

ROS can also directly regulate the autophagy pathway. Scherz-Shouval et al. reported that amino acid starvation activates autophagy through ROS, specifically \( \text{H}_2\text{O}_2 \), as a signaling molecule in mammalian cells. Treating cells with an antioxidant such as NAC during starvation leads to the reduction in LC3 lipidation and phagosome formation, suggesting \( \text{H}_2\text{O}_2 \) is an important signal in starvation-induced autophagy. The source of ROS during starvation seems to come from mitochondria and is partially dependent on PI3K activity. However, the authors did not further investigate the mechanism of ROS generation in this context. Notably, the target of starvation-induced ROS is ATG4. ATG4 cleaves LC3 and GATE-16 at the C-terminus so that
LC3 and GATE-16 can be lipidated. ATG4 can further cleave LC3 and GATE-16, and release them from the membrane. Because ATG4 is a serine protease, it is particularly prone to ROS modification. The authors proposed that ROS produced during starvation inhibits ATG4 activity to ensure that lipidated LC3 and GATE-16 stay on autophagosomes and promote autophagy (Scherz-Shouval et al., 2007). These data suggest that ROS is important to both canonical and non-canonical autophagy activation.

1.4.3 Selective autophagy of mitochondria

Selective autophagy is the specific sequestration and degradation of damaged organelles or protein aggregates by the autophagic machinery. Selective autophagy has many substrates, including and not limited to mitochondria (mitophagy), peroxisomes (pexophagy), ER (ERphagy), aggresomes (aggrephagy), and invading bacteria (xenophagy) (Kirkin et al, 2009a). A common theme in many selective autophagy pathways is the ubiquitination of the substrates that are destined for degradation. To facilitate the sequestration of the correct substrates, autophagy adapter proteins bridge the ubiquitinated substrates and the phagophores together (Fig. 1.6A). Many adapter proteins, such as SQSTM1 and NBR1, contain both a ubiquitin binding domain (UBA) and a LC3-interacting region (LIR) for this purpose (Fig. 1.6B) (Kirkin et al, 2009b). By knocking out endogenous autophagy adaptors, Lazarou et al. demonstrated that optineurin and nuclear dot protein 52 are the only adaptors essential for PARK2-mediated mitophagy in mammalian cells (Lazarou et al., 2015).

There are several distinct pathways that selectively degrade mitochondria in eukaryotic cells, some of which are independent of the ubiquitin signaling pathway. Mitochondrial clearance is essential in the maturation process of reticulocyte. During reticulocyte maturation, cells degrade their mitochondria through BNIP3L (Nix)-mediated mitophagy (Schweers et al, 2007). BNIP3L has a C-terminal TM that localizes it to OMM and a LIR for interaction with LC3 (Novak et al, 2010). BNIP3L+/− mice have an increased number of immature red blood cells containing residual mitochondria that are outside the autophagosomes. However, general autophagy in these BNIP3L+/− reticulocytes was unobstructed, suggesting that only the mitophagy pathway was defective (Schweers et al, 2007). Recently, BNIP3L has also been shown to play a role in the activation of mitophagy that relies on ubiquitin and autophagy adaptor proteins (Ding
Another mitophagy adaptor, FUNDC1, mediates highly selective mitophagy under hypoxic conditions. FUNDC1 is embedded in the OMM with three TMs and binds to LC3 through LIR. Over-expressing FUNDC1 causes autophagosomes to engulf mitochondria and a reduction in mitochondrial protein levels. Interestingly, FUNDC1 activity is regulated by its phosphorylation status, which in turn is regulated by oxygen levels within the cells (Liu et al., 2012).

Figure 1.6. Schematic representation of selective autophagy and autophagy adapters.

(A) Autophagy adapters are responsible for targeting substrates to the phagophore. One of the hallmarks of autophagy substrates is the presence of ubiquitin or ubiquitin chains on the surface of the substrates. Autophagy adapters recognize and bind to the ubiquitin with their UBA domain, and bind to LC3 on the phagophore with LIR, serving as a bridge between the substrates and the phagophores. (B) Selective autophagy adapters such as p62 (SQSTM1) and NBR1 share common features. They have a Phox and Bem1p (PB1) domain for oligomerization, an LC3 interacting region (LIR) for LC3 binding, and a ubiquitin-associated domain (UBA) for ubiquitin
binding. Some adapters that are specific for mitochondria, such as BNIP3L and FUNDC1, contain LIR but not UBA. Instead, they have TMs that anchor them on the mitochondria surface. The schematic is not to scale.

1.4.4 PARK2-mediated mitophagy

PARK2-mediated mitophagy is one of the most well studied mitophagy pathways. In 1998, a group reported that the deletion of a gene, which they named “parkin” (PARK2), causes autosomal recessive familial Parkinsonism in two Japanese families (Kitada et al., 1998). Curiously, the authors noted that PARK2 is a RING-E3 ubiquitin ligase and the first 76 amino acids show similarity to the ubiquitin sequence with a conserved lysine residue at the 48th position (RING stands for really interesting new gene). Parkin mutant Drosophila, though viable, showed reduced climbing ability. Detailed examination revealed that these Drosophila have severe muscle degeneration and swollen mitochondria (Greene et al., 2003).

Ten years after the discovery of PARK2, Narendra et al. demonstrated that PARK2 is a mitochondrial E3 ubiquitin ligase. Under normal conditions, PARK2 appears to be dispersed in the cytosol. However, when mitochondria are depolarized by the weak ionophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), for 1 hr, PARK2 is recruited to the mitochondria from the cytosol. Furthermore, when PARK2 is localized to the depolarized mitochondria, it induces mitochondrial fragmentation and the recruitment of mitochondria to LC3-decorated autophagosomes. The authors proposed that mitochondrial clearance by PARK2 may be implicated in the onset of Parkinson’s disease (Narendra et al., 2008).

The major molecular events in mitophagy were characterized in the HeLa human cervical cancer cell line. Curiously, the PARK2 gene is localized within a common fragile site that has been altered in HeLa cells. HeLa cells do not express endogenous PARK2, therefore most experiments were done in PARK2 overexpression conditions (Denison et al., 2003). Due to the overexpression artifact, treating these cells with CCCP for 24 hr leads to a complete clearance of the mitochondria by mitophagy (Okatsu et al., 2010). It is clear that PARK2-mediated mitophagy needs to be strictly regulated in healthy cells in order to prevent unnecessary mitochondrial loss. The activity of PARK2 is controlled by two mechanisms: 1) PARK2 remains in the cytosol when
mitochondria are polarized; 2) while in the cytosol, PARK2 is inactive through an auto-inhibitory mechanism.

1.4.5 PINK1 recruits PARK2 to the depolarized mitochondria

How is PARK2 recruited to the depolarized mitochondria? Because PARK2 does not have a TM or MTS, it is possible that PARK2 binds to other mitochondrial proteins when mitochondrial potential is disrupted. Genetic studies have revealed that PINK1 (PARK6) acts upstream of PARK2 (Clark et al., 2006). PINK1 was first discovered to participate in the PTEN signaling pathway. When PTEN, a tumor suppressor, is ectopically expressed in endometrial cancer cells, there is an increase in PINK1 mRNA levels. However, the function of PINK1 in the PTEN signaling pathway is not fully understood (Unoki & Nakamura, 2001). Mutations in PINK1 were later discovered to cause autosomal-recessive early-onset PD (Valente et al., 2004). PINK1 possesses a MTS at the N-terminus, followed by a TM and a serine/threonine kinase domain (Song et al., 2013).

Several groups independently reported that PINK1 is essential in the process of PARK2 recruitment to the mitochondria (Vives-Bauza et al., 2010; Narendra et al., 2010b; Ziviani et al., 2010). Our current understanding is that under basal conditions, PINK1 is translocated into the mitochondria and is modified by various mitochondrial proteases such as mitochondrial processing protease (MPP), presenilin associated rhomboid-like (PARL), and mitochondrial ATPase associated with diverse cellular activities (m-AAA). Upon import into the mitochondrial matrix, PINK1 MTS is cleaved by MPP, resulting in an intermediate product that is further processed by PARL and m-AAA to generate a C-terminal fragment. The C-terminal cleaved product is released into the cytosol and undergoes proteasome-dependent degradation. When mitochondrial potential is disrupted, PINK1 does not undergo proteolytic cleavage by MPP, PARL, or m-AAA, and becomes stabilized on the mitochondria (Fig. 1.7) (Greene et al., 2012). Even though the sub-mitochondrial localization of PINK1 remains controversial, it is proposed that PINK1 tethers to the translocase of the outer mitochondrial membrane (TOM) complex upon mitochondrial depolarization. PINK1 then recruits PARK2 to the OMM and allows PARK2 to ubiquitinate its substrates. By tagging PINK1 to different organelles, it is clear that PINK1 alone
is sufficient to recruit PARK2 and drive the autophagic degradation of the targeted organelles (Lazarou et al, 2012).

Figure 1.7. Regulation of PINK1 stability is dependent on mitochondrial polarity

(A) Fully translated PINK1 contains a mitochondrial targeting sequence (MTS in pink color) and a transmembrane domain (in green). When mitochondria are polarized and healthy, PINK1 MTS is transported into the matrix, where MPP recognizes and cleaves the MTS. An IMM protease, presenilin-associated rhomboid-like (PARL), cleaves PINK1 at its TM domain. Cleaved PINK1 is retro-translocated back into the cytosol and degraded by the proteasome. (B) When mitochondria are depolarized, PINK1 can no longer access the matrix and is likely to associate with the TOM complex on OMM. Under this condition, full length PINK1 is not cleaved by the mitochondrial protease or by the proteasome.
1.4.6 PARK2 tertiary structure ensures that it is inhibited under basal conditions

Upon the discovery of PARK2-mediated mitophagy, efforts were made to understand how PARK2 activity is regulated. PARK2 belongs to a sub RING-E3-ligase family called RING between RING (RBR). Besides the UBL domain at the very N-terminus, PARK2 has three RING domains and an in-between RING domain (IBR) (Fig. 1.8) (Rankin et al., 2011). Each of the RING domains and the IRB coordinate with two zinc atoms (Wauer & Komander, 2013), however, only RING1 has the classic RING finger motif arrangement, the other RING domains are atypical. RING1 is responsible for binding to E2-ubiquitin conjugating enzyme and RING2 possesses the catalytic cysteine residue (Trempe et al., 2013). In vitro PARK2 auto-ubiquitination assays revealed that under basal conditions, PARK2 ubiquitination activity remains low, implying the existence of an auto-inhibitory mechanism. Chaugule et al. reported that deleting the UBL domain led to an increase in PARK2 auto-ubiquitination activity. Moreover, PARK2 with pathogenic mutations in the UBL domain also showed an increase in auto-ubiquitination activity in vitro. The authors proposed that UBL domain binds to the C-terminus of PARK2 and inhibits PARK2 activity (Chaugule et al., 2011).

Figure 1.8. Schematic representation of PARK2 domains and their functions.

PARK2 has a ubiquitin like (UBL) at the N-terminus, three RING domains, an in-between RING domain (IBR), and a newly-discovered repressor element (in yellow). RING1 has E2 binding activity, while RING2 possesses the E3-ligase activity. Crystal structure reveals that under auto-inhibitory conditions, RING0 binds to RING2 and inhibits its E3-ligase activity. The repressor elements (shown in yellow) binds RING1 to inhibit RING1’s E2 binding activity. The schematic is not to scale.
X-ray crystallography studies support a model that is different from the one provided by the molecular studies. Trempe et al. demonstrated that the RING0 domain, unique to PARK2, binds to the RING2 domain in order to block the catalytic site. Trempe et al. also discovered a motif between IBR and RING2 that binds to RING1 and represses the E2 binding activity called the repressor element of parkin (REP). \(PARK2^{W403A}\), a mutation within the REP, shows increased ability to remove ubiquitin from an E2-conjugating enzyme compared to WT PARK2. W403A mutant is also recruited to the mitochondria faster than that of the WT, suggesting a relationship between PARK2 activation and recruitment to the mitochondria (Trempe et al., 2013).

With slight discrepancies in the role of the UBL domain, both models suggest that PARK2 adopts a “closed” conformation in order to inhibit its own ubiquitin ligase activity under basal conditions. To become active, PARK2 needs to switch to an “open” conformation to allow access of both RING1 and RING2 domains. However, these models did not go into detail explaining what external or internal stimuli trigger the conformational change in PARK2 during mitophagy. There may be other factors that are important in PARK2 activation.

1.4.7 The role of PINK1 phosphorylation in PARK2 activation during mitophagy

Since PINK1 has kinase activity, PINK1 substrates may also play a role in PARK2-mediated mitophagy. Many pathogenic PINK1 mutations exhibit a decrease in or loss of kinase activity (Song et al., 2013), suggesting that PINK1 kinase activity is important in mitochondrial quality control. Chen and Dorn II reported that the recruitment of PARK2 to mitochondria is dependent on the presence of MFN2 in cardiomyocytes. PARK2 and MFN2 interaction is enhanced when PINK1 is overexpressed. More importantly, mutant \(MFN2^{T111A}\) and \(MFN2^{S442A}\) failed interact with PARK2 in the presence of PINK1 (Chen & Dorn II, 2013). These data suggest that PINK1 phosphorylates MFN2 at T111 and S442, and phosphorylated MFN2 may act as a receptor for PARK2 on the OMM. Evidence of MFN2 phosphorylation leading to interaction with an E3 ubiquitin ligase during cellular stress has been shown previously. When U2OS cells were treated with doxorubicin for 4 hr, a chemotherapeutic drug that induces apoptosis, they exhibited mitochondrial fragmentation and phosphorylation of MFN2 at S27 by JNK. Phosphorylated MFN2 is ubiquitinated by a HECT E3-ubiquitin-ligase, Huwe1. The ubiquitination of MFN2
leads to the proteasomal degradation of MFN2 and enhances apoptosis through an unclear pathway (Leboucher et al., 2012).

Another substrate of PINK1 is PARK2. Kondapalli et al. showed that PINK1 phosphorylates PARK2 at residue S65 in the UBL domain both in vivo and in vitro. Through an in vitro ubiquitination assay, the authors demonstrated that with increasing amounts of wild type PINK1 added into the assay system, PARK2 had increasing levels of ubiquitination activity. However, kinase inactive PINK1 could not stimulate PARK2 ubiquitination activity. Importantly, PINK1-dependent phosphorylation of PARK2 only appeared when cells were treated with a mitochondria depolarization agent such as CCCP, FCCP, and valinomycin. These data further emphasized the importance of the PARK2 UBL domain in the regulation of PARK2 activity (Kondapalli et al., 2012). Taken together, phosphorylation of MFN2 and PARK2 can act as external and internal stimuli to activate PARK2 upon mitochondrial depolarization.

1.4.8 The differential roles of mitochondrial dynamics during mitophagy and autophagy

As I described earlier in this chapter, mitochondrial fusion and fission machineries also play an essential role in PARK2-mediated mitophagy. When selective mitophagy is activated, the fusion and fission machineries cooperate with the autophagy pathway to ensure that mitochondrial fragmentation proceeds so that fragmented mitochondria can be engulfed by autophagosomes (Twig & Shirihai, 2011). However, in other scenarios, mitochondria need to be protected from autophagic degradation. During extreme cellular starvation, mitochondria are protected from the initial degradation by autophagy (Gomes et al., 2011; Rambold et al., 2011).

Rambold et al. reported that mitochondria become elongated and interconnected when MEF cells are deprived of amino acids for 2 hr. This quick adaptation of mitochondrial morphology to an external stimulus imply that the process was not regulated at the transcriptional or translational levels, but at the post-translational level. Indeed, the authors found was that DNM1L activity is inhibited by phosphorylation at residue S637. Furthermore, knocking down MFN1, but not MFN2 resulted in little mitochondrial elongation, suggesting that MFN1 is essential in this process. Therefore, the authors proposed that when cells undergo
starvation, DMN1L is inhibited by phosphorylation, leaving mitochondrial fusion unopposed to generate elongated mitochondria (Rambold et al., 2011). However, these authors did not investigate in detail the purpose of mitochondrial elongation during starvation.

Around the same time, Gomes et al. published a report that largely supported the observations made by Rambold et al. Importantly, Gomes et al. offered more insight into the mechanism and purpose of mitochondrial elongation. When cells are treated with a protein kinase A (PKA) inhibitor in conjunction with starvation, the mitochondria can no longer elongate. This suggests that the PKA pathway is involved in the phosphorylation of DNM1L. The authors also reported that during starvation, mitochondrial polarity and ATP production remain high. However, when mitochondrial elongation is prevented (in OPA1−/− MEFs), both mitochondrial ATP production and cell viability decreased significantly. Therefore, the authors proposed that mitochondria elongated during starvation in order to sustain high levels of ATP production and to avoid being degraded by the autophagy pathway (Gomes et al., 2011). It is important to point out that such extreme starvation is not likely to happen to cells under physiological conditions. Hence, the real implication and significance of this phenomenon remains unclear.

1.5 Ubiquitin dynamics of mitochondria

1.5.1 PARK2 has a wide range of substrates on the OMM during PINK1/PARK2-mediated mitophagy

Ubiquitin chains serve many purposes, including but not limited to signaling to proteasomal degradation and autophagic degradation (Kirkin et al., 2009b). MFN1 and MFN2 were the first mitochondrial substrates discovered to be ubiquitinated and degraded in the early phase of PARK2-mediated mitophagy (Gegg et al., 2010; Chan et al., 2011). Numerous groups have tried proteomics approaches to identify the ubiquitinated substrates during PARK2-mediated mitophagy (Chan et al., 2011; Sarraf et al., 2013). Chan et al. performed stable isotope labeling by amino acid in cell culture (SILAC) analysis on mitochondria that are isolated from cells treated
with CCCP. SILAC combines stable isotope labeling with mass spectrometry to determine the identity and relative quantity of proteins on the mitochondria (Chan et al., 2011).

Of all OMM proteins, MFN1 and MFN2 appeared to have the biggest reduction in protein levels during PARK2-mediated mitophagy. Upon CCCP treatment, MFN1 and MFN2 protein levels dropped sharply through proteasomal degradation in cells overexpressing PARK2. Interestingly, Chan et al. found that DNM1L levels on the mitochondria increased dramatically upon CCCP treatment (Chan et al., 2011). These data suggest that mitochondrial fission was left unopposed during mitophagy. In a scenario where only a portion of the mitochondrial network is damaged, unopposed mitochondrial fragmentation ensures that the damaged portion is isolated quickly from the healthy mitochondrial network.

Members of the TOM complex are also PARK2 substrates. TOMM70 undergoes rapid proteasomal degradation upon activation of PARK2-mediated mitophagy (Chan et al., 2011). TOMM70 is one of the major receptors in the TOM complex and it recognizes non-cleavable hydrophobic precursors such as the one possessed by phosphate carrier on the IMM (Neupert & Herrmann, 2007). The degradation of TOMM70 may lead to the disruption of the mitochondrial import process so that the newly synthesized mitochondrial proteins are not imported into a mitochondrion destined for degradation.

Voltage-dependent anion channel 1 (VDAC1) is another well-known PARK2 substrate during mitophagy (Chan et al., 2011). VDAC1 may serve as a binding partner for PARK2 during mitophagy. Knocking down VDAC1 in neuroblastoma cells delays PARK2 recruitment and the clearance of mitochondria by mitophagy upon CCCP treatment. Overexpressing WT VDAC1 can rescue these defects (Geisler et al., 2010). There is a conflicting report suggesting that VDAC1 was not necessary for PARK2-mediated mitophagy. Narendra et al. demonstrated that PARK2 recruitment and mitochondrial clearance were not delayed in VDAC1/3−/− mouse embryonic fibroblast cells (MEFs) compared to WT MEFs (Narendra et al., 2010a). The discrepancy reported by the two research groups could be attributed to the different cell types used in the experiments. However, the role of VDAC1 in mitophagy remains unclear.
1.5.2 PARK2 generates K6, K11, and K63 ubiquitin on mitochondrial upon mitophagy activation

Ubiquitin is a small protein that is covalently conjugated to other proteins to serve as a specific signal. Ubiquitin is conjugated to the substrates by the E1-E2-E3 system (Fig. 1.9). The E1 ubiquitin activating enzyme activates ubiquitin in an ATP-dependent manner and transfers the ubiquitin to the cysteine residue in its active site. E2 ubiquitin conjugating enzyme receives the activated ubiquitin from E1 and prepares to transfer the ubiquitin to E3. There are two types of E3-ubiquitin ligases: HECT E3 ligase and RING E3 ligase. A HECT E3 ligase has the ability to directly bind to ubiquitin and transfer it to substrates. A RING E3 ligase binds to both the E2 enzyme and the substrates, and facilitates the transfer of ubiquitin from the E2 enzyme to the substrates. Therefore, RING E3 ligases do not directly bind to ubiquitin (Kerscher et al., 2006). However, PARK2 is a HECT-like RING E3 ligase, meaning that PARK2 has a catalytic cysteine (Cys431) that can be conjugated to ubiquitin (Wenzel et al., 2011).

Typically, the last glycine residue (Gly76) of ubiquitin forms an isopeptide bond with a lysine residue in the substrates, including another ubiquitin. Ubiquitin can also be covalently conjugated to cysteine and threonine residues. There are 7 lysine residues in ubiquitin: K6, K11, K27, K29, K33, K48, and K63 (Fig. 1.10) (Shaid et al., 2012). Each type of ubiquitin chain adopts a slightly different conformation, and therefore may signal for a different fate of the substrate. K48 ubiquitin chains have a compact “zig-zag” structure and they typically signal for proteasomal degradation of the substrates. K63 chains adopt a straight extended form and they are thought to be the signal for autophagic degradation (Castañeda et al., 2016).

Ubiquitin_Homo_Sapiens

MQIFVKTTLGKTITLEVEPSDIENVKAKIQDKEGIPPDQQRLIFAGKQL
EDGRTLSDYNIQKESTLHLVLRLRGG

Figure 1.9. Amino acid sequence of ubiquitin (Homo sapiens).

Ubiquitin is 76 amino acids long with 7 lysine residues (shaded in grey): K6, K11, K27, K29, K33, K48, and K63. Each lysine residue can be conjugated to another ubiquitin.
E1 ubiquitin-activating enzyme hydrolyse an ATP molecule to activate ubiquitin, the activated ubiquitin is then transferred to the E1 active site. E1 passes the ubiquitin to E2 ubiquitin-conjugating enzyme. There are two types of E3 ubiquitin-ligase, RING-E3 and HECT-E3. HECT-E3 receives ubiquitin from E2 and directly passes on the ubiquitin to the substrates. RING-E3 has binding sites for E2 and the substrate, and facilitates the transfer of ubiquitin from E2 to the substrate. RING-E3 typically does not bind to the ubiquitin directly.

Figure 1.10. E1-E2-E3 ubiquitination pathway.
In the SILAC analysis, Chan et al. observed an increase in K48- and K63-linked ubiquitin chains in mitochondria isolated from cells treated with CCCP. They also confirmed this observation by running isolated mitochondria on SDS-PAGE and immunoblotting with K48- or K63- specific antibodies (Chan et al., 2011). However, Cunningham et al. recently reported that PARK2 is able to generate atypical K6 and K11 ubiquitin chains on the mitochondria. A commercially available K-ɛ-GG antibody can bind to the interface between the substrate and ubiquitin, and immunoprecipitate ubiquitinated peptides. The substrates’ identity and the ubiquitin linkage are revealed by subjecting the peptides to mass spectrometry (Cunningham et al., 2015). However, it is unclear if these PARK2-generated K6 and K11 ubiquitin chains are signaling for proteasome degradation and autophagy, or have completely unique roles during mitophagy.

1.5.3 Phosphorylation of ubiquitin plays important roles in PARK2 recruitment to the mitochondria

Phosphorylation and ubiquitination are traditionally considered two independent modifications of a substrate. Although several groups did report that ubiquitin is phosphorylated at Ser65, the identity of the kinase remained elusive. In 2014, Kane et al. reported that ubiquitin can be phosphorylated by PINK1. To perform a detailed analysis of PINK1 substrates on the mitochondria, CCCP-treated mitochondria were isolated from WT and PINK1 knock out cells. Importantly, to prevent PARK2-dependent protein degradation, HeLa cells that lack endogenous PARK2 expression were used in this experiment. The OMM proteins from the mitochondria were trypsin-digested and were subjected to mass spectrometry. Among all phosphorylated peptides, the authors discovered that mitochondrial ubiquitin is phosphorylated at Ser65. PINK1-dependent phosphorylation of ubiquitin was first confirmed by running ubiquitin that was pre-incubated with or without PINK1 on a phospho-tag gel. Moreover, increasing incubation times with PINK1 led to increasing levels of phosphorylation on ubiquitin. When S65A mutant ubiquitin that cannot be phosphorylated was overexpressed in cells, PARK2 failed to translocate to the mitochondria upon CCCP treatment, implying that phosphorylated ubiquitin may be important to PARK2 activation. Furthermore, PARK2 ligase activity was stimulated by PINK1-phosphorylated-ubiquitin in vitro (Kane et al., 2014).
Using SILAC, Kazlauskaite et al. also discovered that ubiquitin is phosphorylated in the presence of WT PINK1 and CCCP treatment. An in vitro phosphorylation assay revealed that *Tribolium castaneum* PINK1 (TcPINK1) prefers to phosphorylate ubiquitin and the UBL domain of PARK2 over other UBL-containing proteins. In the phosphorylation assay, the authors incubated ubiquitin with various kinases and concluded that only TcPINK1 can phosphorylate ubiquitin. It is known that PARK2 is phosphorylated at Ser65 by PINK1 in order to achieve the active status. The authors observed that in order to achieve the “fully-active” status of PARK2 in vitro, both PARK2 and ubiquitin need to be phosphorylated by PINK1 at residue Ser65. Mutations at Ser65 in either PARK2 or ubiquitin would lead to a decrease or a total inhibition of the ligase activity in PARK2. Kazlauskaite et al. proposed a two-step activation mechanism for PARK2: PARK2 is first phosphorylated at Ser65 in the UBL domain, which leads to a structural change in PARK2 to adopt a primed active conformation; PINK1 then phosphorylates ubiquitin, which binds to PARK2 and keeps it in an active state (Kazlauskaite *et al.*, 2014).

Besides confirming that ubiquitin is phosphorylated by PINK1, Koyano et al. also used an absolute peptide quantification method in combination with mass spectrometry to determine the amount of ubiquitin phosphorylated. They reported that approximately 0.05% of endogenous ubiquitin is phosphorylated by endogenous PINK1 in cells treated with CCCP. Even though the amount of ubiquitin that is phosphorylated is small, it is thought to be enough for PARK2 activation. In contrary to a previous report, when the ubiquitin S65A mutant was overexpressed in cells and mitophagy was activated, Koyano et al. observed that ubiquitin S65A was recruited to the mitochondria by PARK2. These data suggest that phosphorylated ubiquitin only acts as an activator for PARK2, and PARK2 does not exclusively utilize phosphorylated ubiquitin for its ligase activity (Koyano *et al.*, 2014). Given that phosphorylated ubiquitin is important to activate PARK2, more research needs to be done to determine whether phosphorylated ubiquitin play other signaling roles in the cells.
1.6 Deubiquitinating enzymes (DUBs) in mitophagy

1.6.1 Deubiquitinating Enzymes are proteases that remove ubiquitin from substrates

In the previous sub-chapter, I discussed the importance of ubiquitin on mitochondria during PARK2-mediated mitophagy. The dynamics of ubiquitin can also be regulated by deubiquitinating enzymes (DUBs), which are proteases that cleave ubiquitin or ubiquitin-like moieties from selective substrates. The purpose of cleaving the ubiquitin from the substrates is to either recycle ubiquitin for another round of ubiquitination or to alter the fate of the ubiquitinated substrates (Pickart & Rose, 1985; Kim et al., 2011). The human genome database contains 95 putative DUBs, which can be categorized into 5 superfamilies. In terms of catalytic activities, DUBs can be categorized into cysteine protease and metalloprotease. Superfamily Ubiquitin specific protease (USP), Otubain protease (OTU), Machado-Joseph disease protease (MJD), and ubiquitin C-terminal hydrolase (UCH) are all cysteine proteases. Superfamily JAB1/MPN/Mov34 metalloenzyme (JAMM) contains all metalloproteases (Reyes-Turcu et al., 2009).

I’ll focus on the USP superfamily in my thesis, which is the largest superfamily among human DUBs. The signature domain of USPs is composed of a cysteine-histidine-aspartate catalytic triad essential for the protease activities. The cysteine residue in the active site performs nucleophilic attack on the carbonyl group in the peptide bond between ubiquitin and the substrate. The USP forms an intermediate with the ubiquitin, which is then hydrolyzed to free the ubiquitin from the USP. The deprotonation of cysteine is facilitated by the histidine residue and aspartate residue. Although USP members have low protein sequence similarities, the USP catalytic domain remains highly conserved (Daviet & Colland, 2008). However, each USP has a selective set of substrates. This level of specificity is achieved through the structure differences in USPs outside the enzymatic site and the structural differences of different ubiquitin linkages.

1.6.2 DUBs that regulate autophagy

To date, several DUBs are known to regulate the autophagy process. Liu et al. reported that USP10 and USP13 are involved in the membrane nucleation step in the autophagy pathway. The
phagophore membrane nucleation step requires a complex composed of VPS34 (a class III phosphatidylinositol 3-kinase), VPS15, BECN1, and ATG14. Liu et al. discovered a small molecule, Spautin-1, that disrupts autophagosome formation and promotes the degradation of BECN1 within the complex. The authors reasoned that because small molecules tend to be inhibitors rather than activators, Spautin-1 may inhibit DUBs that stabilizes BECN1 in the cells. By systematically knocking down every DUBs in the human genome, Liu et al. discovered that knocking down USP10 or USP13 leads to the degradation of the members in VPS34 complex, while overexpressing USP10 and USP13 leads to a decrease in ubiquitination of BECN1. Addition of Spautin-1 to the cell culture can reverse the effect of USP10 and USP13 overexpression. Surprisingly, the levels of USP10 and USP13 decreased in response to BECN1 or VPS34 knock down, suggesting a complex co-dependent relationship between USP10/13 and the VPS34 complex (Liu et al., 2011).

A20 is a unique DUB that belongs to the OTU superfamily and possesses both deubiquitinating activity and ubiquitin ligase activity. In macrophages, autophagy can be activated by treating the cells with a bacterial endotoxin lipopolysaccharide (LPS). LPS binds to Toll-like receptors on the cell surface and leads to K63-ubiquitination of BECN1 by TRAF6. The ubiquitinated BECN1 is believed to form oligomers and induce an autophagic response to pathogen invasions. Shi et al. reported that when A20 was knocked down with shRNA, BECN1 ubiquitination level increased, implying that A20 may deubiquitinate BECN1. Furthermore, an in vitro deubiquitinating assay revealed that A20 can cleave K63-linked ubiquitin from BECN1. The ubiquitin ligating and deubiquitinating activities are independent of each other in A20; the ubiquitin ligase dead mutant, A20 C624A/C627A was still able to deubiquitinate BECN1 in vivo. Notably, knocking down A20 leads to an increase in LC3 puncta formation in response to LPS treatment. From these data, Shi and Kehrl proposed that A20 is an important negative regulator of LPS-mediated autophagy in macrophages. However, whether A20 can regulate autophagy outside of the immune response remains unclear (Shi & Kehrl, 2010).

1.6.3 DUBs that regulate PARK2 during mitophagy

PARK2 has the ability to auto-ubiquitinate. Even though auto-ubiquitination is considered a sign of PARK2 activation, the purpose of ubiquitin on PARK2 remains unknown. To understand the
regulatory role of ubiquitin on PARK2 activity, Durcan et al. conducted a knock down screen to look for DUBs that affect PARK2 activity. The authors found that knocking down USP8 delays PARK2 recruitment to mitochondria, suggesting USP8 may affect PARK2 activity. Furthermore, USP8 can cleave the preassembled ubiquitin on PARK2 in vitro. To identify the ubiquitination sites on PARK2, the authors overexpressed GFP-PARK2 in U2OS cells, treated cells with CCCP, and immunoprecipitated GFP-PARK2 for LC-MS/MS analysis. Upon CCCP treatment, PARK2 was only ubiquitinated in the UBL domain. Curiously, PARK2 was ubiquitinated at K27, K48, and K76, and the type of ubiquitin linkage was predominantly K6-linked ubiquitin. Durcan et al. overexpressed UB^K6R mutant in cells depleted of USP8 and reported that PARK2 recruitment became unobstructed. Currently, it is believed that PARK2 builds K6 ubiquitin chains during auto-ubiquitination and the K6 ubiquitin chains delay PARK2 activation as a negative feedback mechanism. USP8 deubiquitinates PARK2 in order to promote PARK2-mediated mitophagy. Durcan et al. did not discuss whether the ubiquitin conjugated to PARK2 is phosphorylated (Durcan et al., 2014).

Another DUB, Ataxin-3, a MJD member, has also been shown to alter the ubiquitination status on PARK2. In an auto-ubiquitination assay in vitro, the addition of Ataxin-3 reduces the level of ubiquitination on PARK2, suggesting that Ataxin-3 may be able to deubiquitinate PARK2. Curiously, Ataxin-3 could only remove mono-ubiquitin but not ubiquitin chains from PARK2. These observations led the authors to conclude that Ataxin-3 can only modify PARK2 auto-ubiquitination while these ubiquitin chains are being generated. Next the authors hypothesized that Ataxin-3 may be affecting the discharging of ubiquitin from E2 to PARK2. Indeed, when incubating Ataxin-3 or PARK2 alone with UBC7 (an E2 conjugating enzyme), UBC7 retained the ubiquitin through the thioester bond. However, when Ataxin-3, PARK2, and UBC7 were added together in the reaction, ubiquitin was discharged from UBC7, which coincided with the ubiquitination of Ataxin-3. Therefore, the authors hypothesized that Ataxin-3 forms a complex with PARK2 and E2, and diverges the ubiquitin from PARK2 onto itself while PARK2 performs auto-ubiquitination (Durcan et al., 2012). However, the biological significance of the phenomenon remains unknown.
1.6.4 Ubiquitin specific protease 30 (USP30)

Previous to my thesis work, USP30 was the only known mitochondrial DUB with unclear functions. Among the 16 ubiquitin-specific proteases in *Saccharomyces cerevisiae*, there is one known mitochondrial DUB, Ubp16p. DUBs play an important role in the fusion events of OMM in yeast. Therefore it is hypothesized that Ubp16p may also regulate mitochondrial dynamics (Kinner & Kölling, 2003). USP30 and Ubp16p share 24% identity in amino acid sequence and structural similarity. USP30 has an N-terminal MTS and a TM, which anchors USP30 to the OMM. USP30 also possesses the highly conserved cysteine and histidine boxes that make up the active site (Fig. 1.11) (Nakamura & Hirose, 2008).

![Peptidase C19 ubiquitin hydrolase](image)

**Figure 1.11. Schematic representation of ubiquitin specific protease 30 (USP30) domains.**

USP30 has an N-terminal mitochondrial TM, a cysteine-box (C-box), and a histidine-box (H-box). The TM anchors USP30 to the OMM. The C-box and H-box are highly conserved across all USP family members. The schematic is not to scale.

In 2008, Nakamura and Hirose performed the initial characterization of USP30 in the context of mitochondrial morphology. The authors found that knocking down USP30 with two different siRNAs led to an elongated mitochondrial network in HeLa cells. It is known that knocking down MFN1/2 leads to mitochondrial fragmentation. When USP30 and MFN1/2 were knocked down together, the authors observed that the mitochondria remain in a fragmented state, implying that mitochondrial elongation in siUSP30 cells rely on the presence of MFN1/2. Interestingly, depletion of USP30 in HeLa cells did not affect the protein levels of any mitochondrial fusion or fission proteins, suggesting that USP30 is not involved in the turnover of these proteins (Nakamura & Hirose, 2008).

Yue et al. in 2014 reported the discovery of a small molecule that inhibits USP30 activity and induces mitochondrial elongation called S3. S3 can only bind to WT USP30, but not the
catalytic-dead $USP30^{C77S}$ mutant, implying that S3 inhibits USP30 through direct binding to the cysteine residue in USP30. Yue et al. was able to confirm most observations made by Nakaruma and Hirose. Notably, Yue et al. also observed that inhibition of USP30 using S3 does not alter the protein levels of MFN1/2; however, S3 alters the ubiquitination levels of MFN1/2. It has been shown that Fzo1p, the yeast orthologue of mammalian MFN1/2, requires cycles of ubiquitination and deubiquitination to carry out the membrane fusion process. Yue et al. hypothesized that USP30 may be involved in mitochondrial fusion, specifically MFN1/2-mediated OMM fusion (Yue et al., 2014).

1.6.5 Ubiquitin specific protease 35 (USP35)

Even less is known about USP35. According to the NCBI database, full length USP35 is a relatively large protein, consisting of 1018 amino acids. It has the conserved cysteine box (C450) and histidine box (H862) shared by all USP members (Fig. 1.12A) (Pruitt et al., 2014).

Interestingly, my lab has obtained an isoform of USP35, termed s-USP35, which is 608 amino acid long (accession: bc131489) and has not been previously characterized. An online MTS prediction program, Mitoprot, predicted that the first 54 amino acids of s-USP35 is a cleavable MTS (Claros & Vincens, 1996). A close analysis of the mRNA of the s-USP35 revealed that it is a spliced isoform of the full-length USP35 (l-USP35). The open reading frame (ORF) of s-USP35 transcript is initiated out-of-frame from l-USP35 in order to generate the unique MTS. After 145 base pairs from the initiation ATG, there is a 4-basepair insertion (GTAG) that is unique to s-USP35. The 4 extra base pairs allow the s-USP35 to become in frame with l-USP35 (Fig. 1.12B). The C-terminal portion of s-USP35 (amino acids 51 to 604) and the C-terminal portion of l-USP35 (amino acids 465 to 1018) are 100% identical. Curiously, s-USP35 does not have a cysteine box, implying that it may be catalytically inactive.
Figure 1.12. Schematic representation of s-USP35 and l-USP35

(A) The long isoform of ubiquitin specific protease 35 (l-USP35) contains the highly conserved C-box and H-box. The short-isoform of USP35 (s-USP35) only has the H-box, but not C-box. s-USP35 has a unique MTS that l-USP35 does not possess. However, the C-terminal portions of s-USP35 (from 51st to 604th a.a.) and l-USP35 (from 465th to 1018th a.a.) are identical. (B) In order to generate the unique MTS in s-USP35, the ORF of s-USP35 is initiated out-of-frame from l-USP35 at the 145th base pair. Four extra base pair unique to s-USP35 (GTAG colored in red) are inserted into the transcript of s-USP35, allowing the s-USP35 to become in frame with l-USP35.

Even though USP35 was not previously characterized, a few groups have reported that USP35 is in a breast cancer amplification hotspot. The gene encoding USP35 is located on chromosome 11. Bocanegra et al. performed genomic profiling of 172 breast tumors and identified a focal amplification at 11q14.1. This region contains only 4 genes, including ALG8,
KCTD21, USP35, and GAB2. GAB2 is a known oncogene that increases cell proliferation in normal breast epithelial cells. Therefore, the authors hypothesized that USP35 was simply amplified in breast cancer due to its proximity to GAB2 in the genome (Bocanegra et al., 2010). Recently, a single-nucleotide polymorphism within the locus containing USP35 and GAB2 was identified to be associated with a testicular germ cell tumour (Litchfield et al., 2015).

Interestingly, there is a conflicting report suggesting that USP35 protein and mRNA levels decrease in breast cancer and lung cancer tissues. Liu et al. reported that microRNA let-7a, a tumor suppressor, enhances USP35 expression in a human prostate cancer cell line. Overexpressing l-USP35 led to a decrease in cell viability and proliferation in two cancer cell lines. In search for how USP35 regulates cell proliferation, the authors observed that l-USP35 pulled down A20-binding of NF-κB 2 (ABIN2) and that USP35 stabilized ABIN2 levels by deubiquitinating ABIN2. ABIN2 inhibits tumour necrosis factor alpha (TNFα)-mediated NF-κB activation, hence USP35 may also inhibit NF-κB activation. Indeed, overexpressing USP35 caused a slight but significant decrease in NF-κB activation induced by TNFα (Liu et al., 2015).

These data shed some light on the role of USP35 in cellular activities, mostly viability and proliferation. However, there was no report of the involvement of USP35 in mitochondrial quality control or mitophagy. This is not surprising, considering that only s-USP35 isoform has the capacity to be recruited to the mitochondria.

1.7 THESIS RATIONALE AND HYPOTHESIS

While research on PINK1 and PARK2 provided us with insight into how cells degrade unwanted mitochondria, we have little understanding of how this process is activated and regulated. In the context of regulation, I hypothesized that upon activation, cells have mechanisms to control the rate and the scale of PARK2-mediated mitophagy. PARK2 can ubiquitinate a wide range of OMM proteins and it is important for cells to protect healthy mitochondria from being degraded. One mechanism the cell may utilize is controlling the amount of ubiquitin on the mitochondrial surface by deubiquitinating OMM proteins. Therefore, I hypothesized that there are
mitochondrial DUBs that counteract PARK2 activity either by deubiquitinating PARK2 substrates or by interfering with PARK2-substrate interactions.

In the context of mitophagy activation, we do not know the role of ROS in PARK2-mediated mitophagy. Currently, the most commonly used method to activate mitophagy is to treat tissue culture cells with ionophores such as CCCP or FCCP. CCCP and FCCP can effectively assimilate mitochondrial damage by quickly dissipating the mitochondrial membrane potential. However, these methods did not provide any information on whether mitochondrial ROS can induce mitochondrial depolarization and mitophagy. Moreover, they lack spatial and temporal specificity. I hypothesized that a sharp increase in mitochondrial ROS production can damage the mitochondria and causes mitochondrial depolarization, and I intended to use mtKR as a novel tool to induce mitochondrial damage and activate PARK2-mediated mitophagy.
Chapter 2

2 MATERIAL AND METHODS

2.1 Reagents

2.1.1 Plasmids

The USP30-HA and USP35-HA plasmids were constructed from the open reading frame (ORF) of USP30 and s-USP35 was purchased from the SPARC BioCentre, Hospital for Sick Children®. USP30 ORF and USP35 ORF were C-terminally tagged with a triple HA epitope using the Gateway® recombination system (LifeTechnologies, 12535-019).

The l-USP35 contained a 1242 base pairs (bp) extension compared to s-USP35 (BC131489.1) at the N-terminus. To generate l-USP35, we synthesized the first 1530 bp with a FLAG-tag at the N-terminus (pBMH-USP35N plasmid (Biomatik)). l-USP35 was constructed by subcloning the N-terminus half from pBMH-USP35N and the C-terminus half from psUSP35 into a modified version of the mammalian expression vector pcDNATM3.1+ (LifeTechnologies, V790-20) using complementary restriction sites. In this modified version, the BspHI restriction sites were removed by Klenow treatment. To generate ΔMTS-s-USP35-HA plasmid, primers were designed to replace the first 162 bp of s-USP35 cDNA with a BglII site. To ensure the amplification of ΔMTS-s-USP35, a Kozak sequence was inserted to the 5’ of ΔMTS-s-USP35 cDNA.

The Cerulean-PARK2 (Cer-PARK2) plasmid was constructed by replacing the YFP in the YFP-PARK2 plasmid with monomerized Cerulean using the common NheI and BsrGI restriction sites. The YFP-PARK2 plasmid was generously provided by Richard Youle (NIH, Bethesda). Mitochondria-targeting GFP (mtGFP) was constructed by replacing YFP in the mtYFP plasmid with a monomerized version of EGFP (Clontech: 6085-1). The mtYFP plasmid and mEGFP-N1 plasmid were digested by BamHI and BsrGI.

To construct the RG-OMM™ (mCherry-GFP-OMP25TM) plasmid, we first replaced EGFP on the pEGFP-OMP25TM plasmid with mCherry from the mCherry-C1 plasmid and generated mCherry-OMP25TM. Next, we digested mCherry-OMP25TM and EGFP-N1 with
BglII and BamHI and inserted the EGFP to the C-terminus of mCherry to construct mCherry-GFP-OMP25TM (RG-OMP25).

The plasmid of mitochondria-targeting KillerRed (mtKR) was purchased from Evrogen (cat# FP964). CytoKR and KR-SKL Mitochondria-targeting GFP (mtGFP) was constructed by replacing YFP in the mtYFP plasmid with a monomerized version of EGFP (Clontech: 6085-1). The mtYFP plasmid and mEGFP-N1 plasmid were digested by BamHI and BsrGI.

The Clontech mtRFP plasmid was a gift from Richard Youle (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD). GFP-UB was purchased from Addgene. GFP-LC3 was kindly provided by Dr. Yoshinori Ohsumi (National Institute of Basic Biology, Okazaki, Japan). LAMP1-GFP was obtained from Dr. George Patterson (National Institutes of Health, Bethesda, MD). SOD2-GFP was a generous gift from Andre Melendes (Albany Medical College).

2.1.2 siRNA

siRNA against s-USP35 and l-USP35 (5’-AGUCAGACACGGGCAAGAUUGTT-3’), and siRNA against USP30 (5’-CUAGUCAACACAACCCUAAACUTT-3’) were purchased from GenePharma®. siRNA against USP20 is a pool of two siRNA oligos (5’-CGUGCUGUGCUCAGGA-3’ and 5’-GGACAAUGCUACCCUA-3’) that were purchased from Sigma®. siRNA against DRP1 (5’-AACGCAGACGCGGAAAGAGTT-3’) was purchased from Santa Cruz Biotechnology INC. (sc43732). A pool of three siRNA against OPA1 1: (5’-GAACAGCUCGAAGCUCAUUTT-3’), 2: (5’-GCAAUGGGAUGCAGCAUUUTT-3’), and 3: (5’-GCAAUGGAUGUGUCUUGUUTT-3’) was purchased from Santa Cruz Biotechnology INC. Two siRNA against PINK1 1: (5’-GGAGAUCCAGGCAAUUUUUUTT-3’) and 2: (5’-CCGGACGCUGUUCGUCAUTT-3’) were synthesized by GenePharma Co. Ltd.
2.1.3 Antibodies

The antibodies used in immunoblotting, immunofluorescence, and immunoprecipitation were:
rabbit-anti-PINK1 (Novus Biologicals, BC100-494), mouse-anti-HA (Covance, MMS-101R),
rabbit-anti-HA (SantaCruz, sc-805), mouse-anti-ATP5A (Abcam, 15H4C4), mouse anti-GFP
(Roche, 11814460001), rabbit anti-GFP (Gift from Dr. R.S. Hegde, MRC, UK), mouse-anti-
FLAG M2 (Sigma, F1804), and HRP-goat-anti-GAPDH (Novus Biologicals, NB300-328H).

2.2 Cell Cultures

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (HyClone, SH3008101)
supplemented with 2 mM L-Glutamine (HyClone, SH30034.01) and 10% FBS
(LifeTechnologies, A12617) at 37 °C and 5% CO₂. For live-cell imaging, cells were grown in 4-
well LabTek chamber slides (Thermo Fisher Scientific, 155383). Cells were changed into CO₂-
independent medium (LifeTechnologies, 18045) prior to live-cell imaging. For long-term time-
lapse experiments, 1X antibiotic cocktail (Wisent, 450-115-EL) was added to the CO₂-
independent medium to prevent bacterial growth.

2.3 Transfection

Plasmids were transfected with Lipofectamine-2000 (LifeTechnologies, 11668-019) according to
manufacturer’s instructions 16 to 24 hr prior to treatment. 6 hr to 16 hr after transfection,
Lipofectamine-containing media was replaced with regular growth media to allow cells to
recover. siRNA was transfected with Lipofectamine-2000 according to manufacturer’s
instructions at 72 hr and 48 hr prior to treatment. To ensure that cells have time to recover,
Lipofectamine-containing media was replaced with regular growth media.

2.4 Microscopy

All fluorescent imaging was performed on a Zeiss LSM710 equipped with a 63X 1.4 NA oil
immersion objective and the appropriate lasers. The images were acquired in 1024×1024 pixels
at the depth of 12 bits. For visual presentation only the brightness was adjusted. GFP and Alexa488 signal was acquired using a 488 nm Argon laser with a 493-565 nm or 515-565 nm (in presence of mCerulean) bandpass filter. RFP and Alexa 561 were acquired using a 561 nm diode laser with a 600-700 nm bandpass. mCerulean images were acquired using a 405nm diode laser with a 450-495 nm bandpass filter. All quantification was done using the imaging software Volocity® 6.3 (Perkin Elmer).

Visualization of the mtKR signal was acquired with the 561 nm laser. The pre-pb images were acquire with the 561 nm laser at 1% output. However, due to photo-bleaching of the mtKR, the remaining signal of mtKR could only be visualized with a higher laser output. In this case, the post-pb images were acquired with 2% laser output.

2.5 Immunoblotting

Cells were washed twice with PBS and lysed with cold lysis buffer (0.1M Tris HCl, 0.01% SDS, pH9) with 1X Protease Inhibitor Cocktail (BioShop, PIC002.1). Cell lysates were incubated in 95°C for 15 min and were centrifuged at 15000 rpm for 15 min. Immunoblotting samples were run on 10% or 12% SDS-PAGE and were transferred to PVDF. Membranes were blocked in 5% skim milk in PBST (1x phosphate-buffered saline, 0.05% Tween-20). Membranes were incubated with primary and HRP-conjugated secondary antibodies (concentration according to the manufacturer) in 2% skim milk in PBST. Proteins were visualized with enhanced chemiluminescence detection kit (Froggabio, 20-500-500).

2.6 Immunofluorescence

Cells on cover slips were fixed with 3.7% paraformaldehyde in growth media at 37 °C for 15 min, and washed twice with warm PBS. When digitonin treatment was required, cells were treated with 20 – 40 µM digitonin in HKM buffer for 2 min before the fixing step (ref). Cells were then incubated with 0.1% Triton-X in PBS for 15 min and blocked in blocking buffer (1% FBS (LifeTechnologies, 12483-020) in PBS) for 30 min. Cells were incubated with primary antibodies (concentration according to the manufacturer direction) in blocking buffer for 2 hr or
overnight. Cells were incubated with Alexa Fluor® secondary antibodies in blocking buffer for 1 hr at room temperature. The cover slips were mounted on fluorescent mounting media (DAKO Canada, 34538) on glass slides and dried overnight.

2.7 Quantitative PCR

After 72 hr siRNA treatment, cells were washed with 1X PBS for two times. RNA was isolated from cells using SV Total RNA Isolation system (Promega, Z3100) according to manufacturer’s directions. cDNA was generated from RNA with iScript™ cDNA synthesis kit (BIO-RAD, 1708890) according to manufacturer’s directions. Quantitative PCR was performed using a TaqMan® Real-Time PCR assay, following the protocol suggested by manufacturer (Life Technologies, 4444557).

2.8 Immunoprecipitation

HeLa cells were transfected with FLAG-l-USP35 and s-USP35-HA using X-tremeGENE 9 (Roche, 06365779001) for 24 h. Cells were washed with cold PBS and lysed in 200 µl lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (v/v) NP-40, 2 mM Na₃VO₄, 5 mM NaF, protease inhibitor cocktail). After clearing the lysate at 16000xg for 10 min at 4 °C, 2 µg mouse anti-FLAG antibody or anti-GFP antibody was added to the lysate for 2 hr at 4 °C while shaking. Antibody-bound proteins were precipitated using Protein A-Agarose (Sigma, P2545) for 2 hr at 4 °C. Beads were washed three times using lysis buffer (without protease inhibitors) and proteins eluted with SDS sample buffer and boiling at 95 °C for 8 min.

2.9 mCherry-GFP-lysosome (RG) assay

HeLa cells that were grown in LabTek chambers were transfected with Cer-PARK2 and mCherry-GFP-(RG)-OMM™ 16 to 24 hr prior to the experiment. Cells were imaged on a Zeiss LSM710 equipped with a 63× X 1.4NA oil immersion objective and the appropriate lasers. The
images were acquired in 1024×1024 pixels at the depth of 12 bits. For time-lapse imaging, multiple locations were established with Zeiss imaging software, and the cells were kept at 37°C and CO2-independent media. After the initial images were acquired (0 min), CCCP, E-64, and leupeptin were added to the media. Cells were imaged at 30 min intervals for up to 12 h.

Quantification of the RG assay was done with image analysis software Volocity® 6.3. The area of the cell was determined by drawing a region of interest (ROI) around individual cells, in which the total pixel intensity of green and red signal of each mitochondrion and the area of each mitochondrion were measured. The mitochondrion with its total red signal 1.5 times higher than its total green signal is considered a “red mitochondrion” and hence co-localized with the lysosome. The area of red mitochondria and the total area of mitochondria were calculated and tabulated. Three independent trials were performed, and 22 cells were quantified in each treatment. The percentage of mitochondria that co-localized with the lysosome in each cell was calculated and plotted in a box-plot (n=66). Statistical analysis was done by Sigmaplot™. Rank-sum test was performed on cells treated with siRNA and the control cells. The usual p=0.05 threshold was applied to label differences as “significant”

2.10 Mitochondrial clearance assay

A HeLa cell line stably expressing GFP-PARK2 and mito-DsRed (GPRM) was generously provided to us by the Youle Lab. GPRM cells grown on cover slips were transfected with USP30-HA, s-USP35-HA, or ΔMTS-s-USP35-HA for at least 16 h. Cells were then treated with 10 µM CCCP for 24 h. Cells were fixed with 3.7% paraformaldehyde at 37 °C for 15 min, and were washed with warm PBS twice. Cells were permeabilized with 0.1% Triton-100 for 15 min at room temperature, followed by blocking buffer for 30 min. Cells were then incubated with primary antibody (concentration according to the manufacturer direction) for 2 hr or overnight. Alexa Fluor® secondary antibodies in blocking buffer was then added to the cells and incubated for 1 hr at room temperature. The cover slips were mounted on fluorescent mounting media (DAKO Canada, 34538) on glass slides and dried overnight. To quantify mitochondrial clearance, we counted the cells that had cleared their mitochondria in each treatment. We considered a cell mitochondrial-negative, when there was very little residual mito-DsRed signal left and GFP-PARK2 showed cytosolic localization. Importantly, the residual mito-DsRed signal
should not co-localize with GFP-PARK2, which indicated that the mito-DsRed signal resided in lysosomes and was not a PARK2-positive-mitochondrial-structure. The percentage of cells with no mitochondria was calculated in each treatment. The percentage of untransfected cells with no mitochondria was also calculated and plotted.

2.11 PARK2 recruitment assay

GPRM cells previously described were grown on cover slips and transfected with s-USP35-HA or USP30-HA for 16 to 24 hr prior to treatment. To activate mitophagy, 10 µM CCCP was added to the growth media, and cells were fixed at 0, 30, and 60 min after CCCP treatment. The immunofluorescence and microscopy procedures were described above.

Quantification of the PARK2 recruitment assay was done with Volocity® 6.3. The area of the cell was determined by manually drawing an ROI around individual cells. The Mander’s coefficient of the GFP signal on the RFP signal was measured and tabulated. Student’s t-test was performed and the usual p=0.05 threshold was applied to label differences as “significant”.

2.12 Inducing mitophagy with mtKR

To photo-bleach mtKR or mtRFP, the target region was drawn on the field view of the monitor and the Definite Focus system on the Zeiss LSM 710 was used to maintain focus on a single focal plane. The 561 nm imaging laser was set to 1% output power prior to the photo-bleaching process, and was maintained at 1% or increased to 2% output after photo-bleaching in order to continue visualizing the mitochondria using the mtKR fluorescence signal. When MTRED (Molecular Probes™, M22425 ) was used, the imaging laser power was kept at 1% throughout the experiment. 561 nm laser light at 100% output was used to photo bleach mtKR and mtRFP, and the number of iterations was indicative of the number of times the laser bleached the defined region. Images were taken immediately before and after photo-bleaching the cells, and at the specified time points. For time-lapse experiments, multiple locations in the chamber were selected and saved with Zeiss ZEN imaging software. Each location was imaged at 5 - 10 min intervals.
2.13 ROS detection

To measure the increase in ROS levels after photo-bleaching, cells were incubated in 10 µM OxyBURST® Green H2DCFDA (ThermoFisher Scientific, D2935) at 37°C for 15 min. To prepare for the live-cell imaging, cells were washed with 37°C PBS once and were replenished with CO₂-independent medium. To determine the total fluorescent intensity of the OxyBURST signal within the cell an open pinhole (9.1 µm) was used when acquiring the fluorescent signal from OxyBURST. Photo-bleaching was conducted as described above and images were acquired. The area of the cell was determined by drawing a Region of Interest (ROI) around individual cells, in which the total pixel intensity of the green signal was measured using the image analysis software Volocity® 5.0 (PerkinElmer) in arbitrary units. The total green signal was measured in the pre-pb images and post-pb images. For each cell, the percentage increase was calculated as the increase in total OxyBURST signal post-pb over the total OxyBURST signal in pre-pb image.

2.14 PARK2 requirement quantification (PARe-Q)

To quantify PARK2 recruitment to mitochondria, we used Volocity® to determine the area of PARK2 positive mitochondria. A ROI was used to identify and select Cer-PARK2 positive structures above a pre-determined threshold level in each cell in the 90 min post-pb image. Due to the different expression levels of Cer-PARK2 in each cell, the minimal threshold for the Cer-PARK2 signal was adjusted accordingly to ensure that all PARK2 aggregates were recognized. To measure the background area due to auto-fluorescence, the same threshold was used to measure the area of auto-fluorescence in the pre-pb image. The auto-fluorescence area was then subtracted from the PARK2 recruitment area to obtain the “calculated PARK2 recruitment area”. To measure the area of mitochondria, we measured the area of mtKR that was above a given threshold in the pre-pb image. The total signal of mtKR was obtained by measuring the total pixel intensity of the red channel in the pre-pb image. The percentage of PARK2-positive mitochondria was calculated by the ratio of “calculated PARK2 recruitment area” over “total area of mitochondria”. The percentage of PARK2-positive mitochondria was plotted against its corresponding total mtKR signal in a scatter plot.
We considered each experimental protocol to generate a population of cells, and each cell within the population to constitute an independent observation. For all PARK2 recruitment quantifications, the ratio of “the percentage PARK2 recruitment area” over “total mtKR signal” of each treated cell was ranked and compared to the corresponding ratios of “standard” protocol respectively the control protocols. Calculations were performed with the R statistical workbench using the Wilcox test function and a normal approximation for the calculation of p-values. The usual p=0.05 threshold was applied to label differences as “significant”.
Chapter 3

3 THE ROLE OF USP30 AND USP35 IN PARK2-MEDIATED MITOPHAGY


Ms. Miluska Jauregui performed the immunofluorescence of 1-USP35 and s-USP35. Dr. Mauro Serrichio performed the co-immunoprecipitation of 1-USP35 and s-USP35, and the co-immunoprecipitation of PARK2 and MFN2. I collaborated with Ms. Tasha Stoltz on the PARK2 recruitment analysis.

3.1 INTRODUCTION

Even though the major components in the PARK2-mediated mitophagy are known, how this pathway is regulated remains unclear. Chan et al. demonstrated that PARK2 ubiquitinates a wide range of OMM proteins upon mitophagy activation by CCCP treatment. The recruitment of PARK2 to the mitochondria causes an increase in K48 and K63 ubiquitin accumulation on the mitochondria by 4 and 8 times respectively, suggesting that ubiquitin may play a role in the mitophagy pathway (Chan et al., 2011). Recently, Kane et al. reported that PINK1 phosphorylates ubiquitin on PARK2 and on OMM proteins, and the phosphorylated ubiquitin is essential in mitophagy (Kane et al., 2014). However, little is known about the regulation of ubiquitin dynamics on mitochondria. Cornelissen et al. reported that USP15 counteracts PARK2 activity during mitophagy. Knocking down USP15 leads to an increase in mitochondrial degradation while overexpressing USP15 reduces PARK2-mediated mitochondrial ubiquitination. However, USP15 does not directly affect the ubiquitin ligase activity or the stability of PARK2 (Cornelissen et al., 2014).
More is known about DUBs that directly affect the E3-ubiquitin ligase activity of PARK2. Ataxin-3 has been shown to reduce the auto-ubiquitination activity of PARK2. Surprisingly, Ataxin-3 did not deubiquitinate PARK2 like a classic DUB, rather it stabilized the interactions between PARK2 and its E2 ligase, UBC7, resulting in a decrease in PARK2 auto-ubiquitination activities (Durcan et al., 2012). Because PARK2 auto-ubiquitination is considered a sign of PARK2 activation and mitophagy initiation, Ataxin-3 may affect PARK2-mediated mitophagy. However, the role of Ataxin-3 in mitophagy remains unclear. Recently, Durcan et al. reported that USP8 cleaves ubiquitin chains from PARK2 and enhances PARK2 activity. USP8 cleaves K6-linked ubiquitin chains generated by PARK2 auto-ubiquitination activity. K6-linked ubiquitin on PARK2 is proposed to impede PARK2 recruitment to the mitochondria. Therefore, USP8 is the first DUB that enhances mitophagy by directly altering the ubiquitination status on PARK2 (Durcan et al., 2014).

To gain more insight into the role of DUBs in mitophagy, the McQuibban lab obtained the open reading frames (ORFs) of 63 human DUBs tagged with GFP. Ms. Riya Shanbhag conducted a DUB localization screen in COS7 cells and identified USP30 and USP35 as mitochondrial DUBs. USP30 has been previously characterized in mammalian cells and may affect mitochondrial morphology under basal conditions (Nakamura & Hirose, 2008). Very little is known about USP35, and its mitochondrial localization has never been reported. Here, I provide evidence that USP30 and USP35 affect PARK2-mediated mitophagy. Using classic and novel mitophagy assays, I demonstrate that USP30 and USP35 delay PARK2-mediated mitophagy through different mechanisms.

3.2 RESULTS

3.2.1 USP30 is localized to the mitochondria

To determine the subcellular localization of USP30, I transiently expressed USP30-HA in HeLa cells and performed immunostaining with HA and TOMM20 antibodies. TOMM20 is a component of the outer-mitochondrial import complex and serves as a mitochondrial marker in these experiments. As expected USP30-HA showed colocalization with TOMM20, indicating
that USP30-HA is localized to the mitochondria (Fig. 3.1A). To determine the localization of USP30 when mitochondria are damaged, I treated the cells with 10 µM CCCP for 2 hr. CCCP is a weak ionophore that quickly dissipates the mitochondrial potential, induces mitochondrial damage, and activates PARK2-mediated mitophagy. In CCCP-treated cells, USP30-HA remained colocalized with TOMM20, demonstrating that USP30-HA localization is not altered by mitochondrial depolarization (Fig. 3.1B).

3.2.2 s-USP35 is only localized to polarized mitochondria

To confirm the localization of s-USP35-HA, I transiently expressed s-USP35-HA in HeLa cells and visualized s-USP35-HA by immunostaining. Under basal conditions, s-USP35-HA showed predominantly mitochondrial localization. Notably, some s-USP35-HA appeared to be cytosolic (Fig. 3.2A). Strikingly, in CCCP-treated cells, s-USP35-HA was localized to the cytosol and did not colocalize with the mitochondria (Fig. 3.2B). This led me to hypothesize that s-USP35 localization is dependent on mitochondrial polarity. Minamikawa et al. have demonstrated that after CCCP treatment, the mitochondrial potential can recover in HeLa cells if they are supplemented with regular growth media (Minamikawa et al., 1999). Therefore, I treated cells transiently expressing s-USP35-HA with 10 µM CCCP for 2 hr, then recovered mitochondrial potential by replenishing the cells with regular growth media for 2 hr. In these cells, s-USP35-HA is again localized to the mitochondria (Fig. 3.2C), suggesting that s-USP35-HA only localizes to polarized mitochondria.
Figure 3.1. Analysis of USP30 localization.
HeLa cells transfected with USP30-HA for 16 hr were treated with either (A) 10 µM CCCP or (B) vehicle control DMSO for 2 hr. These cells were then fixed with 3.7% paraformaldehyde and immunostained with anti-TOMM20 (red) and anti-HA antibodies (green). Scale bar represents 10 µm.
Figure 3.2. Detailed analysis of s-USP35 localization

(A-B) s-USP35-HA was expressed in HeLa cells for 16 hr. Cells were then treated with (A) 10 µM CCCP or (B) vehicle control DMSO for 2 hr. Cells were then fixed with 3.7% paraformaldehyde and immunostained with anti-TOMM20 (red) and anti-HA (green) antibodies. (C) s-USP35-HA was expressed in HeLa cells for 16 hr and cells were treated with 10 µM CCCP for 2hr. In order to recover mitochondrial potential, these cells were washed with 1X Dulbecco’s phosphate-buffered saline (DPBS) and were incubated in regular growth media for 2 hr. Cells were then fixed with 3.7% paraformaldehyde and immunostained with anti-TOMM20 (red) and anti-HA (green) antibodies. Scale bar represents 10 µm.
3.2.3 l-USP35 localization is affected by s-USP35

Unlike s-USP35, l-USP35 does not have an N-terminal MTS. When I expressed l-USP35 alone in HeLa cells, it was localized to the cytosol (Fig. 3.3A). Since USP35 has two isoforms, I wondered whether s-USP35 and l-USP35 interact with each other. Dr. Mauro Serricchio in the McQuibban lab performed co-immunoprecipitation using the two isoforms. We found that when coexpressed in HeLa cells, s-USP35 and l-USP35 coimmunoprecipitated under basal conditions and during CCCP treatment (Fig. 3.3B), suggesting that s-USP35 and l-USP35 may form a complex within the cell. Next, we wanted to determine whether s-USP35 can alter the localization of l-USP35. Mrs. Miluska Vissa performed immunostaining of s-USP35-HA and FLAG-l-USP35 in HeLa cells (Fig. 3.3C). Under basal conditions, we observed that s-USP35-HA was colocalized with FLAG-l-USP35, presumably on the mitochondria. Both s-USP35-HA and FLAG-l-USP35 appeared cytosolic during CCCP treatment. Together, these results showed that s-USP35 forms a complex with l-USP35 and the USP35 complex dynamically associates with mitochondria based on the mitochondrial membrane potential. However, because we overexpressed both s-USP35 and l-USP35, it is possible that this accumulation of the USP35 complex on the mitochondria is a result of overexpression.

3.2.4 The localization of s-USP35 is affected by PARK2 during mitophagy

The dynamic association of the USP35 complex with the mitochondria suggest USP35 may localize to the mitochondria by associating with OMM proteins. Since OMM proteins, such as MFN1/2 and VDAC1, are rapidly degraded in a PARK2/proteasome-dependent manner upon mitophagy activation, I was also interested in the localization of USP35 in the presence of PARK2. Because HeLa cells do not express endogenous PARK2 due to exon alteration (Denison et al, 2003), I transiently expressed Cerulean-PARK2 (Cer-PARK2) with s-USP35-HA in HeLa cells. s-USP35-HA was localized to the mitochondria while PARK2 was localized to the cytosol in DMSO-treated cells (Fig. 3.4). As expected, in CCCP-treated cells, PARK2 was recruited to the mitochondria while s-USP35-HA was dissociated from the mitochondria. However, in cells with recovered mitochondrial potential, s-USP35-HA could not localize to the mitochondria decorated with PARK2. These data suggest that s-USP35-HA localization can be affected by PARK2 during mitophagy.
Figure 3.3. The expression of s-USP35 affects the localization of l-USP35

(A) FLAG-l-USP35 was cotransfected with mitochondrial-targeting RFP (mtRFP) for 16 hr in HeLa cells. Cells were fixed with 3.7% paraformaldehyde and immunostained with anti-FLAG antibody. (B) FLAG-l-USP35 and s-USP35-HA were transfected individually or cotransfected into HeLa cells for 24 hr. Cells were then treated with CCCP or DMSO for 2 hr. Cleared cell lysates were immunoprecipitated for FLAG tagged proteins using anti-FLAG antibody and protein A-agarose beads. Protein contents were analyzed on SDS-PAGE and immunoblotted with anti-FLAG and anti-HA antibodies. (C) HeLa cells were cotransfected with FLAG-l-USP35 and s-USP35-HA and then treated with CCCP or DMSO for 2 hr as indicated. Before fixation, cells were treated with digitonin to remove the cytosolic signal of l-USP35. In CCCP treated cells, digitonin treatment was avoided due to the cytosolic localization of s-USP35 and l-USP35. Fixed cells were immunostained with anti-FLAG and anti-HA antibodies. Scale bar represents 10 μm.
Figure 3.4. The localization of s-USP35 is affected by PARK2 during recovery

HeLa cells were transfected with s-USP35-HA and Cer-PARK2 for 24 hr. Cells were then treated with 10 µM CCCP or DMSO for 2 hrs. For the recovery condition, cells were first treated with 10 µM CCCP for 2 hrs, washed once with 1X DPBS, and were incubated with normal growth media for 2 hrs. Cells were treated with 3.7% paraformaldehyde and immunostained with anti-TOMM20 and anti-HA antibodies. Scale bar represents 10 µm.

3.2.5 USP30 affects ubiquitination of OMM proteins by PARK2

As a first test to determine whether mitochondrial DUBs affect PARK2-mediated mitophagy, I knocked down USP30 with siRNA (siUSP30) in a HeLa cell line stably expressing GFP-PARK2 and mtDsRed, referred to as GPRM cells in this thesis. In order to assess whether mitophagy was altered, I measured the abundance of OMM proteins during PARK2-mediated mitophagy (Fig. 3.5A). Consistent with previous reports, MFN2 and VDAC1 were rapidly degraded upon the activation of PARK2-mediated mitophagy (Chan et al., 2011). Densitometry quantification showed that MFN2 levels in siUSP30 cells were significant lower in comparison to cells treated
with control siRNA (siCTRL) at both 1 and 3 hr CCCP treatment (Fig. 3.5B). There was no significant difference in VDAC1 levels between siUSP30 and siCTRL cells (Fig. 3.5C). Furthermore, I observed significantly higher levels of TOMM20 mono-ubiquitination in siUSP30 cells compared to siCTRL cells (Fig. 3.5D). The knock down of USP30 was confirmed by quantitative PCR (qPCR) (Fig. 3.5F). Therefore, USP30 may delay the degradation and ubiquitination of some PARK2 substrates during mitophagy.

Figure 3.5. USP30 affects PARK2-mediated OMM protein degradation

(A) HeLa cells stably expressing GFP-PARK2 were treated with siRNA against USP30 (siUSP30) on day 1 and day 2, and were allowed to recover on day 3. Cells were treated with 10 µM CCCP for 0, 1, 3, and 6 hr. cell lysates were examined for MFN2, VDAC1, and ubiquitinated-TOMM20 levels by immunoblotting. * indicates non-specific bands. (B-E) The average of normalized density of MFN2, VDAC1, unmodified TOMM20 and mono-ubiquitinated TOMM20 was plotted (n=3, P<0.05). (F) RNA was extracted from the cells treated with siUSP30 and siCTRL, and was reverse-transcribed into cDNA. qPCR was performed with primers specific for USP30 cDNA. Primers against GAPDH were used as the control. Compared to the cells treated with siCTRL, cells treated with siUSP30 had 21±2.8% of USP30 RNA remaining (n=3).
3.2.6 USP35 affects MFN2 levels under basal conditions

I have also assessed the degradation and ubiquitination of PARK2 substrates in USP35 knock down cells. Knocking down the USP35 complex led to distinct results compared to USP30. Before CCCP treatment, *siUSP35* cells had a significantly lower MFN2 level compared to *siCTRL* cells (Fig. 3.6A and B). However, this did not affect mitochondrial morphology under basal conditions (See Fig. 3.9C). VDAC1 levels were comparable in *siUSP35* and *siCTRL* cells (Fig. 3.6A and C). Even though the mono-ubiquitinated TOMM20 levels appeared to be higher in *siUSP35* cells, the difference was not statistically significant (Fig. 3.6A and D). The knock down of both USP35 isoforms was confirmed by qPCR (Fig. 3.6F). These data suggest that USP35 has less effect on OMM proteins during mitophagy, but it may play an important role in MFN2 protein homeostasis.

3.2.7 USP20 does not affect ubiquitination of OMM proteins by PARK2

As a control, I also treated cells with siRNA against *USP20* (*siUSP20*). USP20 was identified as a cytosolic DUB in the localization screen. When over-expressed in HeLa cells, Ms. Riya Shanbhag observed that USP20-GFP was diffused within the cytosol and did not colocalize with ATP5A, another mitochondrial marker (Fig. 3.7A). I found that knocking down USP20 did not alter the levels of MFN2 or VDAC1 before or during mitophagy (Fig. 3.7B, C, and D). Unexpectedly, *siUSP20* cells had slightly lower levels of mono-ubiquitinated TOMM20 compared to *siCTRL* cells during mitophagy (Fig. 3.7B and E). The knock down of USP20 was confirmed by qPCR (Fig. 3.7G). Taken together, USP30 and USP35 may have specific effect on OMM protein degradation during mitophagy and under basal condition, but not USP20.
Figure 3.6. USP35 affects PARK2-mediated mitophagy

(A) HeLa cells stably expressing GFP-PARK2 were treated with siRNA against USP35 (siUSP35) on day 1 and day 2, and were allowed to recover on day 3. Cells were treated with 10µM CCCP for 0, 1, 3, and 6 hr. Cell lysates were examined for MFN2, VDAC1, and ubiquitinated-TOMM20 levels by immunoblotting. * indicates non-specific bands. (B-E) The average of normalized density of MFN2, VDAC1, unmodified TOMM20 and mono-ubiquitinated TOMM20 was plotted (n=3, P<0.05). (F) RNA was extracted from the cells and was reverse-transcribed into cDNA. qPCR was performed with primers specific for USP35 cDNA. Primers against GAPDH were used as the control. Compared to the cells treated with siCTRL, cells treated with siUSP35 had 33.3±2% of USP35 RNA remaining (n=3).
Figure 3.7. USP20, a cytosolic DUB, does not affect PARK2-mediated mitophagy

(A) HeLa cells were transfected with USP20-GFP for 24 hr. Cells were fixed and immunostained with anti-ATP5A and anti-GFP antibodies. (B) HeLa cells stably expressing GFP-PARK2 were treated with siRNA against USP20 (siUSP20) on day 1 and day 2, and were allowed to recover on day 3. Cells were treated with 10µM CCCP for 0, 1, 3, and 6 hr. cell lysates were examined for MFN2, VDAC1, and ubiquitinated-TOMM20 levels by immunoblotting. * indicates non-specific bands. (C-F) The average of normalized density of MFN2, VDAC1, unmodified TOMM20 and monoubiquitinated TOMM20 was plotted (n=3, P<0.05). (G) RNA was extracted from the cells and was reverse-transcribed into cDNA. qPCR was performed with primers specific for USP20 cDNA. Primers against GAPDH were used as the control. Compared to the cells treated with siCTRL, cells treated with siUSP20 had 32.9±1.6% of USP20 RNA remaining (n=3).
3.2.8 USP35 affects MFN2 protein and mRNA levels

It is surprising that knocking down USP35 leads to a drastic reduction in MFN2 levels, so I closely examined the unmodified and ubiquitinated MFN2 levels during the early stages of mitophagy. In siUSP35 cells, unmodified MFN2 levels were much lower than those of siCTRL cells. Furthermore, ubiquitinated MFN2 was not detectable by MFN2 antibody in siUSP35 cells at 30 and 60 min of CCCP treatment (Fig. 3.8A). Protein levels can be regulated at two levels: 1) the synthesis and degradation of the protein; 2) the synthesis and degradation of the mRNA. Preliminary data suggested that MFN2 was not degraded by the proteasome in siUSP35 cells. Therefore, I decided to measure the quantity of MFN2 cDNA in siUSP35 cells. Interestingly, the cDNA levels of MFN2 were significantly lower in both siUSP35 cells (Fig. 3.8B). To ensure that siRNA against USP35 (siUSP35 #1) did not produce an off-targeting effect, I acquired a different siUSP35 (siUSP35 #2) and found that MFN2 cDNA quantity also decreased significantly. These data suggest that USP35 may affect MFN2 homeostasis at the mRNA level.

![Image](image.png)

**Figure 3.8. Knocking down USP35 leads to a decrease in MFN2 mRNA and protein levels**

(A) HeLa cells were transfected with siUSP35 on day 1 and day 2, and were recovered on day 3. On day 4, cells were treated with CCCP for 0, 30, and 60 min. Cell lysates were collected and the MFN2 levels were analyzed with immunoblotting. GAPDH levels were also immunoblotted as a loading control. (B) Cells treated with two different siUSP35 were lysed and the mRNA was collected. RNA was then converted to cDNA by reverse transcription. The relative quantity of USP35 and MFN2 were determined with specific primers. (n=3, p<0.05).
3.2.9 USP30 and USP35 delay mitochondrial delivery to the lysosomes during mitophagy

I examined whether USP30 and USP35 complex can affect the rate of mitophagy, which is the rate that cells deliver mitochondria to the lysosomes for degradation. Here, I developed a new technique to quantify the percentage of mitochondrial area undergoing mitophagy in live cell similar to an assay developed in the Kim lab to measure pexophagy. Ms. Yulia Nartiss generated a reporter construct in which two fluorescent proteins mCherry and mGFP in tandem are tagged with the transmembrane domain of synaptojanin 2 binding protein (SYNJ2BP) (herein referred to as RG-mitoOMM) (Fig. 3.9A). When localized to the OMM and facing the cytosol, both mCherry and mGFP are fluorescent, resulting in yellow signals. However, due to the difference in their pKa, the fluorescent signal of mGFP, but not mCherry is quenched in a low pH environment such as the lysosome. The mitochondria localized in the lysosomes only emit the fluorescent signal from mCherry. I can accurately quantify the area of ‘red-only’ mitochondria and the area of total mitochondria in order to determine the percentage of mitochondria in lysosomes.

I co-expressed RG-mitoOMM and Cer-PARK2 in HeLa cells and imaged these cells at 0 and 6 hr of CCCP treatment. Before CCCP treatment, mitochondria appeared yellow in siCTRL, siUSP30, and siUSP35 cells, suggesting knocking down USP30 or USP35 does not induce mitophagy (Fig. 3.9 B, C, and D). Six hr after CCCP treatment, red mitochondria were visible in all treatments (Fig. 3.9 B, C, and D). Quantification revealed that on average, both siUSP30-(24.0%) and siUSP35-treated (27.6%) cells had significantly higher percentages of mitochondria in lysosomes compared to siCTRL cells (14.3%) (Fig. 3.9 E). Hence depleting cells of USP30 and the USP35 complex accelerates PARK2-mediated mitophagy.
3.2.10 USP30 and USP35 delay mitochondrial clearance by mitophagy

During the end stages of mitophagy, mitochondria are degraded by lysosomal hydrolases. It has been shown that 90% of HeLa cells overexpressing PARK2 had no detectable mitochondria-like structures or mitochondrial protein staining 48 hr after CCCP treatment (Narendra et al., 2008). To determine whether USP30 and USP35 affect the end stages of mitophagy, I expressed USP30-HA or s-USP35-HA in GPRM cells and treated these cells with CCCP for 24 hr (Fig. 3.10A). To identify cells that were expressing the DUB protein, I stained cells with an anti-HA antibody. In untransfected control cells, 62.6±2.8% of the cells had cleared their mitochondria. When overexpressing USP30 or s-USP35, 43.2±5.9% and 32.8±7.2% of the cells had cleared their mitochondria respectively (Fig. 3.10B).

Since the s-USP35 dissociates from the mitochondria after CCCP treatment, I wondered whether the MTS at the N-terminus of s-USP35 is important for its function. s-USP35 lacking its MTS (s-USP35ΔMTS-HA) is localized to the cytosol both under basal conditions and during CCCP treatment (Fig. 3.10C). Expressing s-USP35ΔMTS-HA also significantly reduced the percentage of the cells with no mitochondria to 39.5±5.5% (Fig. 3.10D and E). These data, along with data obtained from the RG-assay (Fig. 3.9), suggest that USP30 and USP35 can delay PARK2-mediated mitophagy. Interestingly, USP35 may play additional roles in the cytosol to delay PARK2-mediated mitophagy.

Figure 3.9. USP30 and USP35 delay PARK2-mediated mitophagy

(A) A Schematic representation of the mCherry-GFP-lysosome assay. The ORF of mCherry and GFP in tandem is tagged with an outer mitochondrial membrane-targeting transmembrane domain (RG-mitoOMM). When localized in the cytosol and autophagosomes, mCherry and GFP are both fluorescent, resulting in yellow mitochondria. In a low pH environment such as the lysosomes, only the signal of GFP is quenched, resulting in red mitochondria. (B to D) HeLa cells treated with control siRNA (siCTRL) (B), siRNA against USP30 (siUSP30) (C) or siRNA against USP35 (siUSP35) (D) were cotransfected with RG-mitoOMM and Cer-PARK2 (not shown) for 16 h. Cells were treated with CCCP, E-64, and leupeptin, and were imaged live at 0 hr and 6 hr of the treatment. (E) Quantification of mCherry-GFP-lysosome assay for siUSP30 and siUSP35 versus siCTRL. The total area of mitochondria and the area of red mitochondria in each cell was measured with imaging software Volocity®6.3. The percentages of mitochondria in lysosomes of cells from each treatment were plotted in box plots, and a rank-sum significance test was performed. (n=66 per treatment * P<0.05). Scale bar represents 10 µm.
3.2.11 USP30, but not USP35 delays PAKR2 recruitment to the mitochondria

In cells with polarized mitochondria, PARK2 is diffuse in the cytosol and not colocalized with the mitochondria. Following mitophagy activation, PARK2 is recruited to the mitochondria within 1 hr. USP30 and USP35 may delay PARK2 recruitment to the mitochondria in order to delay mitophagy. Ms. Tasha Stoltz helped me perform some of the following experiments. We overexpressed USP30-HA or s-USP35-HA in GPRM cells and treated them with CCCP for up to 60 min. Colocalization of PARK2 and mitochondria was measured by Manders colocalization coefficient. In mock transfected cells, the average Manders coefficient of PARK2 on mitochondria at 30 and 60 min after CCCP treatment was 0.59±0.03 and 0.85±0.06 respectively. In contrast, cells overexpressing USP30 showed significant reduction in PARK2 recruitment at 30 min (0.07±0.06) and 60 min (0.64±0.04) (Fig. 3.11). s-USP35 overexpressing cells did not show any delay in PARK2 recruitment compared the mock. This indicates that USP30 impedes PARK2 recruitment during the early stages of mitophagy, resulting in delay in PARK2-mediated mitophagy. By contrast, the USP35 complex may delay PARK2-mediated mitophagy through a different mechanism, likely through the regulation of MFN2 levels.

**Figure 3.10. USP30 and USP35 delays mitochondrial clearance mediated by PARK2**

(A) GPRM cells were transfected with USP30-HA or s-USP35-HA for at least 16 h. Cells were treated with 10 µM CCCP for 24 hr prior to fixing with 3.7% paraformaldehyde and immunostained with an anti-HA antibody. (B) The percentage of the cells with cleared mitochondria was calculated for each treatment (n=3, P<0.05). (C) HeLa cells transiently expressing s-USP35ΔMTS-HA were treated with vehicle control DMSO or 10 µM CCCP for 2 hr. Cells were fixed with 3.7% paraformaldehyde and immunostained with anti-TOMM20 and anti-HA antibodies. (D) GPRM cells were transfected with s-USP35ΔMTS-HA for 24 hr. Cells were then treated with 10 µM CCCP for 24 hrs before being fixed and immunostained with anti-HA antibody. (E) The percentage of the cells with cleared mitochondria was calculated for each treatment (n=3, P<0.05). Scale bar represents 10 µm.
Figure 3.11. USP30, but not s-USP35 delays PARK2 recruitment to the mitochondria during mitophagy

GPRM cells were transfected with USP30-HA, or s-USP35-HA for 16 hr. Cells were then treated with 10 μM CCCP and fixed after 30 min or 60 min. Cells were treated with digitonin for 2 min prior to fixation in order to remove nontargeted GFP-PARK2. Cells were stained with anti-HA antibody in order to visualize the cells expressing DUBs. Manders coefficient of GFP-PARK2 on mito-DsRed was measured with Volocity®6.3. The average of Manders coefficient was shown (n=3, P<0.05).

3.2.12 USP30 and USP35 interfere with the PARK2-MFN2 interaction

MFN2 is one of the early PARK2 substrates during mitophagy. MFN2 is degraded in a PARK2/proteasome-dependent manner within 1 hr of mitophagy activation. Moreover, phosphorylated MFN2 on the mitochondria may serve as a receptor for PARK2 during mitophagy. To assess whether PARK2-MFN2 interaction can be altered by USP30 and the USP35 complex, Dr. Mauro Serricchio knocked down USP30 and USP35 in GPRM cells and performed immunoprecipitation of GFP-PARK2. Under basal condition, we observed some MFN2 co-immunoprecipitated with PARK2 in siCTRL cells. However, both siUSP30 and siUSP35 cells showed stronger interaction between PARK2 and MFN2 compared to siCTRL cells. Upon CCCP treatment, we observed an increase in PARK2 and ubiquitinated MFN2 association. Importantly, PARK2 pulled down more ubiquitinated MFN2 in siUSP30 and siUSP35 cells in comparison to that in siCTRL cells (Fig. 3.12). These data suggest that USP30...
and USP35 can interfere with PARK2-MFN2 interaction under basal conditions and during CCCP treatment.

**Figure 3.12. USP30 and USP35 prevents PARK2-MFN2 interactions during the early stages of mitophagy**

GPRM cells were transfected with siCTRL, *siUSP30*, and *siUSP35* for 72 h. Cells were treated with 10 µM CCCP or DMSO for 30 min. PARK2 was immunoprecipitated from the cell lysates with anti-GFP antibody and Protein A-sepharose beads. The content of the immunoprecipitation was analyzed by SDS-PAGE and immunoblotting with anti-PARK2 and anti-MFN2 antibodies.

### 3.3 DISCUSSION

#### 3.3.1 USP30 is a negative regulator of PARK2-mediated mitophagy

I have demonstrated that USP30, a mitochondrial DUB, regulates PARK2-mediated mitophagy at several levels. First, USP30 may directly oppose PARK2 activity by deubiquitinating OMM proteins. I have shown that depleting USP30 in HeLa cells leads to a faster degradation and ubiquitination of some OMM proteins during PARK2-mediated mitophagy (**Fig. 3.5 A, B, & D**). The notion that USP30 can counteract PARK2 activity by directly deubiquitinating PARK2...
substrates during mitophagy is supported by other research groups (Bingol et al., 2014; Cunningham et al., 2015). Cunningham et al. demonstrated that USP30 prefers to remove K6- and K11-linked chains from the mitochondria that are built by PARK2. Furthermore, using K-GG immunoaffinity purification and mass spectrometry, the authors identified several USP30 substrates from the OMM including TOMM20 and VDAC1. Curiously, they did not identify MFN2 as a USP30 substrate (Cunningham et al., 2015). It is possible that upon mitophagy activation, MFN2 was degraded so rapidly that it was difficult to detect ubiquitinated MFN2 by this technique.

Secondly, USP30 may delay mitophagy by delaying PARK2 recruitment to the mitochondria. I demonstrated that in cells overexpressing USP30, PARK2 recruitment to the mitochondria was reduced compared to the mock transfected cells (Fig. 3.11). Moreover, stronger PARK2-MFN2 interaction was observed in siUSP30 cells (Fig. 3.12). Previously published literatures suggested that PARK2 activation depends on PINK1-mediated phosphorylation and binding to the phosphorylated MFN2. MFN2 mutants that cannot be phosphorylated fail to co-immunoprecipitate with PARK2. In MFN2−/− cardiac myocytes, PARK2-mediated mitophagy is defected upon FCCP treatment (Chen & Dorn II, 2013). My data are in agreement with the previously published observations, providing an alternative mechanism for the regulation of mitophagy. By disrupting the physical interaction between PARK2 and MFN2, USP30 interferes with both PARK2 recruitment and PARK2-dependent ubiquitination on the mitochondria. However, it is still unclear how USP30 disrupts PARK2-MFN2 interactions.

3.3.2 The dynamic association between the USP35 complex and mitochondria

USP35 complex is a novel mitochondrial DUB, which has not been characterized previously. I have shown in this chapter that s-USP35 and l-USP35 form a complex that associates with the polarized mitochondria (Fig. 3.3B & C). However, it is not clear how USP35 localizes to the mitochondria. By Mitoprot prediction, the MTS residing on the N-terminus of s-USP35 is cleavable, implying that s-USP35 may be imported into the matrix (Claros & Vincens, 1996). Because l-USP35 does not have an MTS, it is unlikely that l-USP35 can be imported into the
mitochondria. My data suggest, but cannot prove, that s-USP35 and l-USP35 are on the surface of the mitochondria by binding to certain OMM proteins. First, I showed that s-USP35 dissociated from and re-associated with the mitochondria within 2 hrs of depolarization or potential recovery (Fig. 3.2C). Secondly, over-expressing PARK2 prevents s-USP35 from re-associating with the mitochondria, suggesting that USP35’s binding partner may be a substrate of PARK2 during mitophagy (Fig. 3.4).

Since mitochondria are a signaling hub responsible for many processes in mammalian cells, there are examples of other proteins dissociating from the mitochondria due to environmental stimuli. Hexokinase II (HK2) is a glucokinase localized to the OMM through binding with VDAC. HK2 participates in the first step of glycolysis. It is known that HK2 dissociates from the mitochondria when the energy source of the cells is switched from glucose to galactose. The detachment of HK2 from the mitochondria can be linked to cytochrome c release and cell death (Chiara et al, 2008). A recent paper has suggested a link between HK2 activity and PARK2 recruitment to the mitochondria (McCoy et al, 2014).

Through immunoprecipitation, I have shown that s-USP35 and l-USP35 physically interact with each other (Fig. 3.3B). Because s-USP35 is localized to the mitochondria and l-USP35 has the full active site, this interaction between s-USP35 and l-USP35 may be necessary to bring the de-ubiquitinating activity of USP35 to the level of mitochondria. There are other examples in the cellular system where a catalytically inactive isoform forms a complex with the active isoforms and modulates the activity of the complex. DNA (cytosine-5)-methyltransferase 3 beta (DNMT3B) is responsible for the initial methylation of DNA in the embryo and is known to form complexes with other DNA methytransferases, such as DNMT3A and DNMT3L. Van Emburgh and Robertson reported that DNMT3B isoform 3 (DNMT3B3) is catalytically inactive but forms complexes with DNMT3A and DNMT3L to promote their methylation activity (Van Emburgh & Robertson, 2011). Taken together, s-USP35 may be able to modify the location and the activity of l-USP35 within the USP35 complex. However, to date, we do not fully know the abundance and the expression patterns of the s-USP35 transcript in human.
3.3.3 Impact in the field

In this chapter, I have identified two mitochondrial DUBs, USP30 and USP35, that affect PARK2-mediated mitophagy. The role of USP30 in mitophagy was reported by other groups in recent years. Given its importance in PARK2-mediated mitophagy, USP30 may become a suitable drug target in neurodegenerative diseases and cancer. The effect of USP35 on mitochondria has not been previously characterized, however it may be important to mitochondrial quality control. My data provide additional information to the field of mitophagy regulation, further implying the roles of DUBs in PARK2-mediated mitophagy.
Chapter 4

4 MITOCHONDRIAL-TARGETING KILLERRED (MTKR) IS A NEW TECHNIQUE TO INDUCE PARK2-MEDIATED MITOPHAGY


Ms. Yulia Nartiss assisted in the initial characterization of mtKR. Dr. Boris Steipe assisted in statistical analysis of PARe-Q results.

4.1 INTRODUCTION

Historically, there is a link between reactive oxygen species (ROS), mitochondrial dysfunction, and neuronal death in PD. In PD patients’ SNpc, there is a reduction in complex I activity in the electron transport chain (Schapira et al, 1988), which leads to an increase in ROS production and further inhibition of the complex I activity. Neuronal cells demand high levels of ATP synthesis (Clarke & Sokoloff, 1999), therefore the oxidative phosphorylation and ROS production are upregulated. ROS can damage mitochondrial DNA, protein, and lipids, and cause a decrease in mitochondrial membrane potential (Gomes & Scorrano, 2012). Frank et al. reported that mild oxidative stress in MEFs can lead to mitochondrial fragmentation and mitophagy (Frank et al, 2012). Mitochondrial depolarization is the key to trigger PINK1 stabilization on the mitochondria and the subsequent PARK2 recruitment (Narendra et al, 2010b). However, the source and the type of ROS that is linked to mitophagy remains unclear.

Although ROS production has been implicated in PARK2-mediated mitophagy (Joselin et al, 2012), the source of ROS produced remains unclear. The most logical production site is
within the mitochondria, but there are only few techniques that mimic the condition in which mitochondrial ROS level rises sharply and induces mitophagy. The most common method to induce mitophagy is treating cells with CCCP, which is a weak ionophore that quickly dissipates the potential of the mitochondria. CCCP effectively depolarizes mitochondria in a short period of time and leads to PARK2 recruitment and mitophagy activation. However, CCCP treatment bypasses the ROS step that is proposed to be important in the context of PD and mitophagy. Moreover, CCCP treatment leads to a global mitochondrial depolarization, which lacks spatial sensitivity. Kim and Lemasters reported that irradiating mitochondria with a strong 488 nm laser can induce the selective degradation of the photo-damaged mitochondria (Kim & Lemasters, 2011). The authors hypothesized that photo-damage may lead to ROS production in the mitochondria and activate mitophagy, but they did not provide direct evidence to support this model.

The other question that has not been answered is the type of ROS that leads to the activation of PARK2-mediated mitophagy. The ROS generated by complex I and III within the mitochondria is superoxide, a highly reactive and membrane-impermeable type of ROS (Orrenius, 2007). Superoxide can damage mitochondrial DNA and lipid, resulting in mitochondrial inner membrane permeability. SOD2, an antioxidant protein residing in the mitochondria, quickly converts superoxide into H$_2$O$_2$. H$_2$O$_2$ is membrane permeable, therefore it can leave the mitochondria and enter the cytosol. Once in the cytoplasm, H$_2$O$_2$ is decomposed by glutathione into O$_2$ and H$_2$O (Scherz-Shouval & Elazar, 2010). It has been shown that both superoxide and H$_2$O$_2$ can upregulate the formation of autophagosomes, hence both types of ROS may be able to activate mitophagy (Chen et al, 2009; Scherz-Shouval et al, 2007).

Here, I introduce a new mitophagy induction technique that will address some of the unanswered questions remaining in the field. By targeting the fluorophore, KillerRed, to the mitochondrial matrix (mtKR), I can induce bursts of superoxide within the mitochondria. With the help of laser-scanning confocal microscopy, I can activate mtKR spatially and temporally. In this chapter, I demonstrate that activating mtKR leads to the production of superoxide within the mitochondria that causes mitochondrial depolarization, PARK2 recruitment, and mitophagy activation. Furthermore, I provide evidence demonstrating that mtKR-induced mitophagy can be inhibited by SOD2 and the process of mitochondrial elongation.
4.2 RESULTS

4.2.1 mtKR activation produces ROS in the mitochondria

I postulated that an acute elevation of ROS level within the matrix of mitochondria can induce mitochondrial damage and activate mitophagy. Previous literatures have shown that mtKR can produce ROS both in vitro and in vivo. To directly demonstrate that mtKR produces ROS in mitochondria, I incubated cells transiently expressing mtKR with a ROS dye, OxyBURST® Green H2DCFDA. Before photo-activation or photo-bleaching (pb), the OxyBURST signal was low and mainly observed in the mitochondria, indicating that they are the major ROS production site in the cells (Fig. 4.1A). In order to visualize mtKR, pre- and post-photo-bleaching, the image was acquire with the 561 nm laser at 1% output. Upon photo-bleaching mtKR with the 561 nm laser light at 100% output for 30 iterations, I observed a decrease in the mtKR signal and an increase in OxyBURST signal (Fig. 4.1A). As a control, I performed the same experiment in cells expressing mitochondria-targeting red fluorescent protein (mtRFP). After photo-bleaching, there was a similar bleaching effect in mtRFP signal; however, the OxyBURST signal remained unchanged (Fig. 4.1B). These data suggest that photo-activation of mtKR leads to photo-bleaching of the mtKR signal as well as production of ROS specific to mtKR.

4.2.2 Activating mtKR leads to long-term photo-bleaching of mtKR

To further characterize the mtKR system, I quantified the mtKR signal before and after photo-bleaching. As shown in Fig. 4.2A, photo-bleaching mtKR with 100% laser output for 30 iterations leads to up to 85% signal reduction. Furthermore, the 561 nm laser does not affect the signal of other fluorophores, such as mitochondrial-targeting green fluorescent protein (mtGFP) (Fig. 4.2A). Continuous monitoring of the cell revealed that upon photo-bleaching, the mtKR signal remained low and did not recover in 100 min after photo-bleaching (Fig. 4.2B).
HeLa cells were transfected with mtKR (A) or mtRFP (B) for 24 hr and incubated with 10 μM OxyBURST® Green H₂DCFDA for 15 min. Cells were then irradiated with 561 nm laser light for 30 iterations at 100% output. Images were acquired before (pre-pb) and after photo-bleaching (post-pb) the cells. The scale bar represents 10 μm.
Figure 4.2. Characterization of mtKR fluorescent profile

(A) HeLa cells expressing mtKR and mtGFP were photo-bleached with 561 nm laser at 100% output for 15 iterations, 100% output for 30 iterations, 1% output for 30 iterations, or 2% output for 30 iterations. The total signal intensity of the red and the green channels of each cell in the pre-pb and the post-pb states were measured. The average percent fluorescent signal loss of 10 cells was calculated for each condition. (B) A HeLa cell expressing mtKR and mtGFP was photo-bleached by 561 nm laser at 100% output for 30 iterations. The cell was continuously imaged for 120 min post-pb. At 105 min, 200 nM MTRED was added to the well containing the photo-bleached cell. The total signal intensity of the red and green channels was measured and graphed.

4.2.3 ROS production by mtKR is controlled by laser power and mtKR level

One of the goals in developing a technique to induce mitophagy by ROS is to strictly control the amount of ROS released by mtKR. I observed that ROS increase is dependent on both the number of iterations during photo-bleaching and the expression levels of mtKR itself. To quantify this observation, I photo-bleached mtKR with the 561 nm laser at 100% for 5, 10, 15, and 30 iterations in the presence of 10 µM OxyBURST in HeLa cells. I quantified the OxyBURST signal before and after photo-bleaching and found that 30 iterations of laser resulted in 25%±5% increase in OxyBURST. mtRFP photo-bleached by 30 iterations resulted in 6%±3% increase in OxyBURST signal (Fig. 4.3A). I also plotted the increase in OxyBURST signal as a function of the total fluorescence intensity of mtKR before photo-bleaching. I found that there
was a strong linear relationship between the total mtKR signal and the increase in OxyBURST signal over the assayed range (Fig. 4.3B). Taken together, these data showed that the amount of ROS produced is determined by the level of mtKR expression and the amount of photo-bleaching.

Figure 4.3. ROS production of mtKR can be controlled by laser iterations and mtKR expressions

(A) The mtKR- and mtRFP-expressing cells were incubated with 10 μM OxyBURST and were irradiated with 561 nm laser light for 5, 10, 15, or 30 iterations. Pre-pb and post-pb images were acquired using an open pinhole. The OxyBURST fluorescent signals in the pre-pb and post-pb images were measured with the image-processing software Volocity® and the average percentage increase in OxyBURST fluorescence signal was quantified from three independent experiments (n=10). (B) HeLa cells expressing mtKR were incubated with 10 μM OxyBURST and irradiated with the 561 nm laser light for 5, 10, 15, or 30 iterations (n=9). The increase in total OxyBURST signal after the photo-bleach was plotted against the corresponding total mtKR fluorescence signal of the cell. Linear regression lines calculated with a zero-intercept are shown with the data points.
4.2.4 mtKR activation leads to PARK2 recruitment to the mitochondria

To test whether ROS produced within the mitochondria leads to PARK2-mediated mitophagy, I examined the recruitment of PARK2 to the mitochondria. I coexpressed Cer-PARK2 and mtKR and photo-bleached mtKR with 30 iterations of 561 nm laser at 100% output. Sixty min after photo-bleaching, mitochondria became fragmented and PARK2 was recruited to the mitochondria (Fig. 4.4A). As a control, I coexpressed Cer-PARK2 and mtRFP and photo-bleached mtRFP with 30 iterations of 561 nm laser at 100% output. As expected, there was no mitochondrial fragmentation nor PARK2 recruitment (Fig. 4.4B). Photo-bleaching mtKR proves to be a feasible technique to activate PARK2-mediated mitophagy, however, I could not rule out the possibility that general increase in cellular ROS may induce mitophagy.

4.2.5 KR in cytosol or peroxisomes does not lead to PARK2 recruitment to the mitochondria

It has been suggested that an increase in cellular ROS or exogenously added ROS can upregulate general autophagy. To ensure that only ROS from the mitochondrial matrix induces PARK2 recruitment, I coexpressed Cer-PARK2 with cytosolic KR (cyto-KR) or peroxisome-targeting KR (KR-SKL) (Fig. 4.5A & B). Photo-bleaching either cyto-KR or KR-SKL did not lead to PARK2 recruitment to the mitochondria 60 min post photo-bleaching, demonstrating that only ROS produced within the mitochondria could recruit PARK2 to the mitochondria.
Figure 4.4. ROS produced by mtKR leads to PARK2 recruitment to the mitochondria

HeLa cells that were transfected with mtKR (A) or mtRFP (B) and Cer-PARK2 16 to 24 hr prior to the experiments. Cells were irradiated with 561 nm laser for 30 iterations at 100% output. Images were acquired immediately before and after the photo-bleaching. Cells were then imaged live for 60 min. The 561 nm laser output used to acquire pre-pb images was set to 1%, and 2% laser output was used to acquire the post-pb images due to photo-bleaching of the mtKR. Scale bar represents 10 µm.
Figure 4.5. ROS produced in the cytosol or peroxisomes does not activate PARK2 recruitment to the mitochondria

(A) HeLa cells were transfected with cyto-KR, Venus-mito<sub>OMM</sub>, and Cer-PARK2 for 24 hr. The white outline shown in the merge panel indicates the region in which the KR was photo-bleached with the 561 nm laser for 30 iterations at 100% output. A pre-pb image and a post-pb image were acquired and the cells were monitored for 60 min after photo-bleaching. Laser power used to acquire pre-pb images was 1%, for post-pb 2%. (B) Similar experiments were done with KR targeted to peroxisomes (KR-SKL). The scale bar represents 10 µm.
4.2.6 A new method to quantify mitophagy activation – PARe-Q

Previously, mitophagy activation was quantified by scoring cells as positive or negative for PARK2 recruitment. These methods suffer from subjectivity and may mask important data. Therefore, I developed a new method to quantify and represent PARK2 recruitment more accurately. Taking the advantage that PARK2 accumulates on the mitochondria during mitophagy activation, we quantified the area of PARK2 accumulation on the mitochondria in relation to total mitochondrial area. From these two values, I calculated the percentage of mitochondria that are PARK2-positive. I also measured the mtKR expression level in each cell before photo-bleaching. To detect the basal level of PARK2 recruitment to the mitochondria, I calculated the percentage of PARK2-positive mitochondria in mtRFP-expressing cells 90 min post photo bleaching. There was no obvious colocalization between PARK2 and mitochondria in these cells. Moreover, quantification showed that cells contain less than 3% PARK2-positive mitochondria 60 min after photo-bleaching mtRFP. Hence, 3% was defined as the basal level of PARK2-positive mitochondria in the subsequent experiments (Fig. 4.6A).

I performed photo-bleaching of mtKR in cells with 5, 10, 15, and 30 iterations of 561 nm laser at 100% output, and found that the percentage of cells with greater than 3% PARK2-positive mitochondria increased with increasing number of laser iterations in these cells (Fig. 4.6B, C, D, & E). Taken together, these data indicate that an increase in ROS production leads to an increase in PARK2 recruitment during mitophagy.

4.2.7 mtKR-mediated PARK2 recruitment depends on PINK1

PINK1 has been shown to accumulate on the depolarized mitochondria and to facilitate the recruitment of PARK2 in order to activate mitophagy. To test if PARK2 recruitment triggered by photobleaching mtKR was dependent on PINK1, I knocked down PINK1 with siRNA in HeLa cells (Fig. 4.7A) and found that PARK2 recruitment was severely inhibited in siPINK1 cells (Fig. 4.7B). Quantification indicated that siPINK1 cells could not activate PARK2 recruitment after photobleaching mtKR, while 58% of siCTRL cells had more than 3% PARK2-positive mitochondria (Fig. 4.7C and D). These data suggest that mtKR-induced PARK2 recruitment is mediated by PINK1.
Figure 4.6. A new method to quantify PARK2 recruitment – PARe-Q

(A) HeLa cells expressing mtRFP and Cer-PARK2 were irradiated with 561 nm laser for 30 iterations at 100% output. The PARK2-positive mitochondrial area over the total mitochondria area was quantified in cells 90 min post-pb using Volocity®. Three percent PARK2-positive mitochondria was defined to be the basal level of PARK2 recruitment to mitochondria ($n=16$). The divide at 3% is shown with a dotted line. (B-E) HeLa cells expressing mtKR and Cer-PARK2 were irradiated with 561 nm laser for 5 (B), 10 (C), 15 (D), or 30 (E) iterations at 100% output ($n=46$ for each set). The percentage of PARK2-positive mitochondria was calculated and plotted against the total mtKR fluorescent signal. Shown on the right of the graph is the percentage of cells with PARK2-positive mitochondria above 3% (data points in the red regions) and those below 3% (data points in green region). The percentage on the right side of each graph indicates the fraction of the cells with mitophagy activation (red) and without mitophagy activation (green).
Figure 4.7. PARK2 recruitment to the mitochondria depends on PINK1

(A) Lysates from HeLa cells treated with either control siRNA (siCtrl) or siRNA against PINK1 (siPINK1) for 72 hrs were separated on a SDS-PAGE and was immunoblotted with antibody against PINK1. The arrows indicate PINK1-specific bands while the asterisks indicate a cross reacting band. (B) Representative image of a HeLa cell that received siPINK1, and was transiently expressing mtKR and Cer-PARK2. The cell was photo-bleached by the 561 nm laser light. Two-hundred nM MTRED was added after photo-bleaching. An image was acquired before, after, and 90 min after photo-bleaching. The laser power was kept at 1% for both pre-pb and post-pb image acquisitions. (C & D) HeLa cells expressing mtKR and Cer-PARK2 were treated with siCTRL (C), or siPINK1 (D) were irradiated with 561 nm laser light for 30 iterations. These cells were imaged again 90 min after photo-bleaching and the percent of PARK2 recruitment was determined (n=29). The difference between the siPINK1 and the siCTRL population is significant (p=5.8×10⁻⁶). The scale bars represent 10 µm.
4.2.8 mtKR activation leads to PARK2-mediated mitophagy

Based on the data on mitophagy with CCCP as the damaging agent, the current view of mitophagy is that once PARK2 is localized to the mitochondria, it ubiquitinates OMM proteins (Youle & Narendra, 2011). Ubiquitinated mitochondria are then engulfed by LC3-decorated autophagosomes, and degraded in the lysosomes (marked by LAMP-1). I examined localization of ubiquitin (UB), LC3, and LAMP-1 following photobleaching mtKR. As shown in Fig. 4.8, all three markers of mitophagy colocalized with PARK2-decorated mitochondria 60 min, 90 min, and 4 hr after photobleaching mtKR. One advantage of selectively activating mitophagy via mtKR is that it allows for more accurate temporal measurement of the molecular events during mitophagy. On average, PARK2 began to appear on the mitochondria 21 min post photobleaching, which was followed by UB (on average 34 min). The initial colocalization between LC3 and mitochondria took on average 41 min after photobleaching (Fig. 4.8D). Even though these steps of mitophagy have been previously described, I have now provided a method to determine the specific timing of each step.

4.2.9 mtKR does not induce mitophagy in the absence of PARK2

As mentioned in the introduction, there are other mechanisms to activate mitophagy in a PARK2-independent manner. To test if mtKR only leads to PARK2-mediated mitophagy, I activated mtKR in HeLa cells without expressing PARK2. As I mentioned in Chapter 3, HeLa cells do not have endogenous PARK2 expression. UB and LC3 did not localize to the mitochondria 60 min and 90 min post photobleaching respectively (Fig. 4.9A & B). Prolonged monitoring demonstrated that LC3 did not colocalize with mitochondria 19 hr after photobleaching, indicating that mtKR-mediated mitophagy requires PARK2 (Fig. 4.9B).
Figure 4.8. Photo-bleaching of mtKR leads to the sequential events of the PARK2-dependent mitophagy pathway.

HeLa cells expressing mtKR, Cer-PARK2, and one of the following constructs: GFP-UB (A), GFP-LC3 (B), or GFP-LAMP-1 (C) were irradiated with 561 nm laser light for 30 iterations and were imaged by time-lapse at 5 min intervals for up to 6 hr. The representative images are a GFP-UB expressing cell at 60 min post-pb (A), a GFP-LC3 expressing cell at 90 min post-pb (B) and a GFP-LAMP-1 expressing cells at 4 hr post-pb (C). (D) Plot of the time when PARK2, Ub or LC3 first show colocalization with the mitochondria. The dash indicates the average time of recruitment (n=14). Cer-PARK2 first appeared on mitochondria at 20.4 min post-pb, 34 min for GFP-UB, and 40.1 min for GFP-LC3. Scale bar represents 10µm.
Figure 4.9. The recruitment of UB and LC3 to mitochondria after photo-bleaching mtKR is a PARK2-dependent process

(A) HeLa cells expressing mtKR and GFP-UB but no Cer-PARK2 were photo-bleached by 561nm laser light for 30 iterations at 100% output. Cells were imaged live 60 min after photobleaching. (B) HeLa cells expressing mtKR and GFP-LC3 were photobleached by 561nm laser light. Cells were imaged live for up to 19 hr after photobleaching. The images shown were acquired at 100 min and 19 hr after photobleaching respectively. Laser power used to acquire pre-pb images was 1%, for post-pb 2%. The scale bar represents 10 µm.
4.2.10 mtKR can induce mitochondrial damage spatially

It is not clear whether mtKR also leads to mitochondrial depolarization or uses a different signaling pathway to initiate mitophagy. Here, I examined the mechanism by which mitochondrial ROS induces mitophagy. To visualize mitochondrial potential after photobleaching mtKR, I used MitoTracker® Red (MTRED), which is a dye that only accumulates in polarized mitochondria. Although MTRED and mtKR have similar excitation/emission spectra, the mtKR fluorescence signal remains low and negligible post photobleaching (Fig. 4.2B). As shown in Fig. 4.10, I coexpressed mtKR and mtGFP in HeLa cells and photobleached a region of mtKR indicated by the white outlines. Thirty min post photobleaching, I added MTRED to the cell to visualize mitochondrial potential. In the photobleached region (Fig. 4.10 zoom in 1), two populations of mitochondria were observed. Arrows point to the population of mitochondria that have both mtGFP and MTRED signal, suggesting that they remain polarized post photobleaching. Arrowheads point to the population of mitochondria that emit only the mtGFP signal, indicating that they were depolarized. In the region that was not photobleached (Fig. 4.10 zoom in 2), there was no mitochondrial fragmentation nor depolarization observed. Therefore, the mtKR technique can be used to spatially and selectively damage mitochondria.

4.2.11 mtKR induces PARK2 recruitment spatially

Next, I tested whether PARK2 is specifically recruited to these depolarized mitochondria. I photobleached a selective region of the mitochondria in a HeLa cell expressing mtKR and GFP-PARK2 (indicated by the white outline in Fig. 4.11A Pre-pb). I then added MTRED to the cell to visualize mitochondrial potential. MTRED staining revealed that the mitochondria located at the lower-left portion of the cell were depolarized (Fig. 4.11A). GFP-PARK2 accumulation was also observed in the same region. Upon close examination, PARK2 positive mitochondria were depolarized, whereas polarized mitochondria did not recruit PARK2 (Fig. 4.11A zoom in). This differential localization of PARK2 and MTRED can be readily seen in the fluorescent intensity line analysis of the image (Fig. 4.11B), which showed that only mitochondria negative for MTRED signal contained signal for GFP-PARK2.
Figure 4.10. mtKR can induce mitochondrial depolarization locally

Representative images of HeLa cells transfected with mtKR and mtGFP and irradiated with 561nm laser light in the region indicated by the white outline (pre-pb). Two-hundred nM MitoTracker® Red (MTRED) was added to the cells after photo-bleaching and the cells were imaged live for 30 min. Two areas indicated by the white box in the 30 min + MTRED panel have been magnified. In zoom 1, the solid arrowheads indicate mitochondria with absent MTRED staining and the arrows indicate mitochondria with both mtGFP and MTRED signal. The laser power was kept at 1% for all imaging acquisitions. Scale bar represents 10 μm.
Figure 4.11. Local activation of mtKR induces local PARK2 recruitment

(A) Representative images of HeLa cells expressing mtKR and GFP-PARK2 and irradiated with 561 nm laser in the region indicated by the white outline (pre-pb). Two-hundred nM MTRED was added to the cells following photo-bleaching and the cells were imaged again for 30 min. The laser power was kept at 1% for all imaging acquisitions. The white square box area is magnified in the panel labeled as “zoom in”. The arrow path in the “zoom in” panel indicates the pixel intensity of the fluorescent signal from GFP-PARK2 (green) and MTRED (red) shown in (B). (B) The pixel intensity of red and green channels was plotted against the pixel position along line scan. The scale bar represents 10 µm.
4.2.12  mtKR-induced mitophagy can be inhibited by the mitochondrial antioxidant protein

Computational simulation studies predicted that KR produces superoxide upon photoactivation, a highly reactive and membrane impermeable ROS (Roy et al., 2010). Superoxide produced within the mitochondrial matrix is converted to hydrogen peroxide ($\text{H}_2\text{O}_2$) by the mitochondrial antioxidant protein, superoxide dismutase 2 (SOD2). To determine which species of ROS is responsible for inducing mitophagy, I overexpressed SOD2 and analyzed PARK2 recruitment after mtKR photobleaching with PARE-Q. Cells overexpressing SOD2-GFP did not show mitochondrial fragmentation, depolarization, or PARK2 recruitment (Fig. 4.12A). In comparison, control cells overexpressing mtGFP exhibited mitochondrial fragmentation, depolarization, as well as PARK2 recruitment 90 min after photobleaching (Fig. 4.12B). PARE-Q analysis confirmed the protective nature of SOD2 since 8% of cells overexpressing SOD2 showed PARK2 recruitment, whereas 37% of control cells showed PARK2 recruitment (Fig. 4.12C & D). These data suggest that over-expressing SOD2 can significantly impede mtKR-induced mitophagy, further implying that superoxide is the ROS responsible for inducing mitophagy.

4.2.13  A SOD2 mimetic, mitoTempo, also inhibits mtKR-induced mitophagy

To confirm that superoxide is the type of ROS that induces mitophagy, I photobleached mtKR in cells incubated with different ROS scavengers that neutralize different ROS species. Cells incubated with mitoTempo, a superoxide scavenger, showed significant reduction in PARK2 recruitment after photobleaching mtKR compared to vehicle control (Fig. 4.13A & B). In contrast, treating cells with NAC, which eliminates $\text{H}_2\text{O}_2$, did not inhibit mtKR-induced mitophagy (Fig. 4.13C). Taken together, these data strongly suggest that superoxide, not $\text{H}_2\text{O}_2$, is the ROS that initiates PARK2-recruitment to the mitochondria.
Figure 4.12. Over-expression of SOD2 impedes ROS-induced mitophagy

(A and B) Representative images of HeLa cells expressing mtKR and Cer-PARK2 with either SOD2-GFP (A), or mtGFP (B) and irradiated with 561 nm laser light for 30 iterations. Pre-pb and post-pb images were acquired. Fifteen min prior to the 90 min post-pb image was taken, 200 nM MTRED was added to the photo-bleached cell. The laser power was kept at 1% for all imaging acquisitions. (C and D) PARE-Q was done on the images of cells treated as in A and B (n=35). 8% of SOD2-GFP expressing cells showed PARK2 recruitment 90 min post-pb (C), while 37% of mtGFP expressing cells showed PARK2 recruitment (D) This difference is significant (p=0.033).
Figure 4.13. Superoxide, but not H2O2 is responsible for mtKR-induced mitophagy

HeLa cells expressing mtKR and Cer-PARK2 were treated with DMSO control (A), 10 nM mitoTempo (B), or 10 mM NAC (C) immediately before imaging. Cells were photo-bleached with 561 nm laser for 30 iterations. Ninety min after the photo-bleach, cells were imaged again. The area of Parkin recruitment and total mtKR signal were measured with Volocity® (see detail in Material and Methods). The percentage of PARK2 positive mitochondria and total mtKR signal of each cell was plotted on a scatter plot. The percentage of PARK2 positive mitochondria and total mtKR signal of each cell was plotted on a scatter plot. (n=34).

4.2.14 Modifying mitochondrial morphology can interfere with mtKR-induced mitophagy

It is known that mitochondrial dynamics play an important role in mitochondrial quality control. Mitochondrial elongation induced by starvation prevents their degradation by general autophagy. Mitochondrial division proteins, such as DRP1, are recruited to the mitochondria and induce mitochondrial fragmentation during mitophagy. To change mitochondrial morphology, I knocked down DRP1 to induce mitochondrial elongation and knocked down OPA1 to induce
fragmentation (Fig. 4.14A & B). Photobleaching mtKR in cells with elongated mitochondria (siDRP1) did not lead to mitochondrial potential loss or PARK2 recruitment (Fig. 4.14A). Conversely, siOPA1 cells had fragmented mitochondria that were much more prone to depolarization and PARK2 recruitment post photobleaching (Fig. 4.14B). PARE-Q analysis further validated my observations (Fig. 4.14C & D). Thus, these data suggested that elongated mitochondria possess some property that makes them more resistant to ROS-induced mitophagy.

4.3 DISCUSSION

4.3.1 Activating mtKR leads to PARK2-mediated mitophagy

In this chapter, I have demonstrated that activating mitochondrial-targeting KillerRed (mtKR) leads to PINK1/PARK2-mediated mitophagy. Irradiating mtKR with a strong 561 nm laser leads to a sharp increase in the ROS level within the mitochondria and the photo-bleaching of mtKR (Fig. 4.1-4.3). This sudden elevation of ROS production causes mitochondria to depolarize and induces PARK2 recruitment to the mitochondria (Fig. 4.4). Subsequently, ubiquitin, LC3, and LAMP1 are recruited to the mitochondria in a sequential manner (Fig. 4.8). Notably, mitophagy induced by mtKR is dependent on PINK1 and PARK2, suggesting that mtKR-induced mitophagy still follows the canonical mitophagy pathway (Fig. 4.7 & 4.9). We now have direct evidence that ROS production within the mitochondria is upstream of mitochondrial depolarization and is able to induce PARK2-mediated mitophagy. Therefore, mtKR provides an alternative method to activate mitophagy and monitor mitophagy in live cells.
Figure 4.14. Mitochondrial morphology plays a role in ROS-induced mitophagy

(A & B) HeLa cells transfected with siRNA against DNM1L (A) or OPA1 (B) for 72 hrs were also transfected with mtKR and Cer-PARK2 during the last 16 hrs before imaging. Cells were irradiated with 561 nm laser for 30 iterations. An image was acquired at pre-pb and at post-pb. 200 nM of MTRED was added to the photo-bleached cells 15 min before the 90 min images were acquired (90min + MTRED). The scale bar represents 10 µm. (C, D, & E) PAr-e-Q on the data collected from DNM1L and OPA1 knockdown experiments (n=44). Twenty-three percent siDNM1L treated cells showed PARK2 recruitment (C) while 80% siOPA1 treated cells were positive for PARK2 recruitment (D). (E) Fifty-one percent of the siCtrl-treated cells showed PARK2 recruitment 90 min after mtKR was photo-bleached. The differences between the siDNM1L and the siOPA1 respectively the siCtrl population are significant (p=2.1x10^{-10} & 6.9x10^{-5}); the difference between the siOPA1 population and the siCtrl population is also significant (p=6.4x10^{-6}).
4.3.2 The advantage of mtKR as a mitophagy-induction technique

Even though CCCP treatment has been widely accepted as the traditional method to induce mitophagy, its damage to the mitochondria is less physiologically relevant to the disease mechanism in PD. Moreover, CCCP treatment leads to global depolarization of mitochondria in all cells and lacks spatial and temporal sensitivities. There are several advantages to using mtKR to induce PARK2-mediated mitophagy: 1) the dose of ROS production can be controlled through mtKR activation; 2) mtKR activation can be controlled spatially with high level of accuracy.

I have shown that the amount of ROS produced by mtKR positively correlates with the number of laser iterations used to photo-bleach mtKR and the mtKR expression level itself. This property of KR provides a more accurate way to control ROS production while studying mitophagy. It has been shown that treating cells with low concentrations of H$_2$O$_2$ or inducing cellular starvation leads to mild oxidative stress and mitophagy (Frank et al, 2012). However, because H$_2$O$_2$ is an important signaling molecule involved in many cellular processes, it is difficult to determine whether mitophagy was activated due to mitochondrial damage by ROS or the upregulation of general autophagy. Although I did not fully explore the potential of dose-dependent mtKR activation, it is possible to induce long-term low-level oxidative stress to the mitochondria by continuously activating mtKR with a weaker laser or with fewer laser iterations.

Transient depolarization is frequently observed in dividing mitochondria. After mitochondrial fission, one of the daughter mitochondria may exhibit loss of mitochondrial potential. If the depolarized mitochondrion does not recover, it will be eliminated by autophagy (Twig et al, 2008). This implies that under basal conditions, mitophagy occurs to a few selective damaged mitochondria. The mtKR technique can be employed to study the cellular response to local mitochondrial damage and depolarization. I showed that mtKR can be activated in a selected region of the mitochondrial network and that mitophagy occurs only to the damaged mitochondria (Fig. 4.10A). In contrast, CCCP treatment cannot provide such spatial specificity. Furthermore, mitophagy is only activated when mtKR is photo-bleached, this helps us record the time of mitophagy events more accurately. Using mtKR, Ashrafi et al. demonstrated that damaged mitochondria in the neuronal axons do not return to the cell body for autophagic degradation. Instead, these damaged mitochondria are degraded in the axons in a PINK1/PARK2 dependent manner (Ashrafi et al, 2014). This observation cannot be reproduced by CCCP
treatment because 1) CCCP depolarizes all the mitochondria within the neuron and 2) mitochondria damaged in the axon are removed very quickly. Taken together, the mtKR technique provides unique advantages in the field of mitophagy and PD.

4.3.3 Superoxide, but not H$_2$O$_2$ is responsible for inducing mitophagy

In the mitochondria, superoxide is produced by complex I and III in the electron transport chain (Orrenius, 2007). Due to its highly reactive nature, superoxide quickly reacts with mitochondrial lipid, protein, and DNA, or it is quickly neutralized by SOD2. ROS serve as signaling molecules that are involved in essential cellular processes such as growth and defence (Huang et al., 2011). I have determined that superoxide, but not H$_2$O$_2$, is the ROS that induces PARK2-mediated mitophagy. Overexpressing SOD2 inhibited the ability of mtKR-produced ROS to activate mitophagy, suggesting that reducing superoxide levels is responsible for impeding mitochondrial depolarization and mitophagy activation (Fig. 4.12). Treating cells with mitoTempo, an antioxidant specific for superoxide, but not NAC (antioxidant specific for H$_2$O$_2$) also led to a reduction in mtKR-induced mitophagy (Fig.4.13).

It is not surprising that superoxide is responsible for inducing mitochondrial depolarization and mitophagy. In a scenario where complex I produces too much superoxide for SOD2 to neutralize, superoxide is trapped in the mitochondria, directly modifying proteins on the inner mitochondrial membrane, and leading to mitochondrial permeability by transition pore opening (Koopman et al., 2010). H$_2$O$_2$, on the other hand, can diffuse into the cytosol where it is neutralized by other antioxidant proteins. Taken together, the type of ROS that induce mitophagy is superoxide, not H$_2$O$_2$.

4.3.4 A potential role of mitochondrial morphology in PARK2-mediated mitophagy

It has long been proposed that mitochondrial dynamics play a crucial role in both mitochondrial quality control and mitophagy (Twig & Shirihai, 2011). My data from this chapter suggest that mitochondrial morphology affects the outcome of mtKR-induced mitophagy (Fig. 4.14A).
Knocking down DNM1L, a mitochondrial fission factor, led to an elongated and inter-connected mitochondrial network. Strikingly, activating mtKR in these mitochondria led to very low levels of mitochondrial depolarization and PARK2 recruitment. DNM1L is heavily involved in the fate of mitochondria under stress conditions. Depending on the stimuli, DNM1L can respond to save mitochondria or promote their degradation. In cells undergoing extreme starvation, DNM1L dissociates from the mitochondria, allowing for unopposed mitochondrial fusion. These elongated mitochondria have increased ATP production and are able to avoid autophagic degradation through an unknown mechanism (Gomes et al., 2011). It is possible that DNM1L is required for mtKR-induced mitophagy, or elongated mitochondria have increased antioxidant activities.

Conversely, knocking down OPA1 led to severe mitochondrial fragmentation. Loss of OPA1 causes inner mitochondrial membrane disruption, a reduction in mitochondrial potential, and mitochondrial fragmentation. As expected, these fragmented mitochondria are more prone to mtKR-induced mitophagy activation (Fig. 4.14B). Although I did not observe any loss in mitochondrial potential in siOPA1 cells prior to mtKR activation, it is possible that these mitochondria are less capable of neutralizing the bursts of superoxide produced by mtKR. These data further strengthen the argument that mitochondrial morphology is important in combating ROS production and maintaining mitochondrial health.

4.3.5 Impact to the field

One of the unanswered questions in the field of mitophagy is how cells selectively eliminate a mitochondrion when it becomes damaged by ROS under basal conditions. In the context of PINK1/PARK2-mediated mitophagy, ROS has been suggested to be the cause of mitophagy activation. However, until now, the type of methods that induce oxidative stress and mitophagy was limited. With mtKR, we can induce oxidative stress spatially and temporally in selective mitochondria and study their fate using live cell imaging. mtKR technique provides a new way to study mitophagy with more spatial and temporal accuracy.
5 GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 Summary

In the past decade, interest in understanding mitophagy has intensified due to its involvement in neurodegenerative diseases such as PD. Thanks to the discovery of the genes involved in familial Parkinsonism, we now have a big picture of the molecular events in PARK2-mediated mitophagy. However, knowledge gaps exist in the activation and the regulation of this pathway.

In my thesis, I first demonstrated that mitochondrial DUBs may play important roles in counteracting PARK2-mediated mitophagy by either directly opposing PARK2 activity or by regulating mitochondrial health. I have shown that USP30 1) opposes the ubiquitination and degradation of PARK2 substrates; 2) delays PARK2 recruitment to the mitochondria; and 3) disrupts interactions between PARK2 and MFN2 during mitophagy. USP35, on the other hand 1) affects MFN2 mRNA and protein levels; 2) disrupts interactions between PARK2 and MFN2 during mitophagy; but 3) does not affect PARK2 recruitment during mitophagy. Currently, we have limited understanding of how DUBs are involved in mitophagy. However, we do know that DUBs, such as USP8 and Ataxin-3, can affect PARK2 auto-ubiquitination and activation (Durcan et al., 2014, 2012). There are other DUBs, such as USP15 and USP30, that oppose PARK2 activity by deubiquitinating PARK2 substrates during mitophagy (Cornelissen et al., 2014; Cunningham et al., 2015). My work, along with previously published data, support a model in which PARK2-mediated mitophagy is regulated by many DUBs at many different levels.

Secondly, in search of a more relevant way to activate mitophagy with ROS, I employed a new photosensitizer, mitochondrial-targeting KillerRed (mtKR), which produces superoxide within the mitochondrial matrix. Furthermore, I have developed two fluorescent-based mitophagy quantification methods to quantify PARK2 recruitment to the mitochondria and the mitochondrial delivery to the lysosomes more accurately. The utilization of mtKR has been adopted in the mitophagy field. Using mtKR, Ashrafi et al. elegantly demonstrated that mitochondria damaged within the axon are degraded without being retracted back to the cell
body (Ashrafi et al., 2014). Wong et al. showed that a mitophagy adapter, optineurin (OPTN), is recruited to the mitochondria that are damaged by mtKR, but not the healthy mitochondria (Wong & Holzbaur, 2014). These data suggest that activating mtKR can be a versatile tool in mitophagy research.

5.2 ARE MITOCHONDRIA THE CAUSE OF OXIDATIVE STRESS IN PD?

There are still major questions remaining in the role of mitochondria in neuronal death in PD. By understanding the precise disease mechanism of PD, we can develop better diagnostic tools and drugs targeting PD. The decline in mitochondrial activity and the increase in oxidative stress are common features in both familial and sporadic PD patients, suggesting a strong link between mitochondrial damage and the onset of PD (Ryan et al., 2015). However, it is unclear whether mitochondrial damage leads to oxidative stress of the cell, or ROS generated from other sources cause oxidative stress in the mitochondria. The discovery of genes involved in familial PD has shed some light on this “chicken or egg” dilemma, but we still cannot fully explain the disease mechanism of PD.

The current data on PINK1 and PARK2 suggest that mitochondrial dysfunction and the accumulation of damaged mitochondria is one of the major causes of neuronal death in PD. In animal models, knocking out PINK1 or PARK2 causes mitochondrial dysfunction in tissues that have high energy demand, such as neurons and muscles (Yao et al., 2012; Palacino et al., 2004). PINK1 or PARK2 mutations can lead to early on-set Parkinsonism, implying the importance of eliminating damaged mitochondria by mitophagy (Kitada et al., 1998; Valente et al., 2004). In addition, PINK1 and PARK2 can systematically remove the mitochondria that are damaged by ROS or depolarization agents (Fig. 4.7 & 4.8). However, this argument does not explain the majority of the sporadic PD cases, in which PD patients have PINK1 and PARK2 without disease-associated mutations (Wang et al., 1999; Healy et al., 2004). It is possible that in the presence of functioning PINK1 and PARK2, the cells can still be overwhelmed by oxidative stress and damaged mitochondria in sporadic PD. Therefore, other factors may also contribute to the pathogenesis in the majority of PD cases.
Another possible source of ROS comes from defects in dopamine storage in the
dopaminergic cells. Upon synthesis, dopamine is stored in synaptic vesicles and is released into
the synapse upon stimulation. However, many environmental factors, such as the uptake of
methamphetamine and SNCA fibril build-up can perturb dopamine storage in the vesicles,
resulting in dopamine release into the cytosol (Lotharius & Brundin, 2002). The formation of
dopamine-o-quinone and H$_2$O$_2$ can induce oxidative stress to the mitochondria and the cell. In
this scenario, damaged mitochondria may further contribute to the ROS production and activate
apoptotic pathways.

One difficulty in deciphering the exact role of mitochondria in sporadic PD is that we
need tools to generate mitochondrial ROS at a level that is physiologically relevant to the cells in
tissues. In chapter 4, I demonstrated that mitochondrial-targeting KillerRed (mtKR) can be used
as a tool to induce mitochondrial depolarization and PINK1/PARK2-mediated mitophagy. As a
proof of concept, I was able to activate global as well as local mitophagy with mtKR (Fig. 4.4 &
4.10). In these experiments, I suspect that mtKR produced high levels of superoxide and directly
triggered the opening of mitochondrial permeability transition pores (Zorov et al., 2006). We do
not yet know if ROS can also act as a signaling molecule to induce mitophagy. MtKR can be
adapted to mimic a condition in which mitochondria generate small amount of ROS for a long
period of time. By titrating the amount of ROS generated by mtKR, we can induce low levels of
mitochondrial or cellular oxidative stress, but not enough to induce global mitochondrial
depolarization, and study the cellular signals in these conditions. One can also study the
localization and activity of PINK1 and PARK2 when mitochondria undergo prolonged, but mild
oxidative stress.

5.3 THE ROLE OF MITOCHONDRIAL DUBS IN PARK2-
MEDIATED MITOPHAGY

Even though it is difficult to generate an inhibitor for a specific DUB, there has been some
success. Recently, inhibitors specific to USP7 and USP8 have been identified in a drug-like
molecule screen. USP7 and USP8 have been shown to be implicated in cancer. The inhibition of
USP7 leads to the stabilization of p53 and the inhibition of USP8 leads to delay in cancer cell
growth (Daviet & Colland, 2008). In recent years, several DUBs were discovered to regulate
PARK2-mediated mitophagy either by directly interfering with PARK2 activity or by deubiquitinating PARK2 substrates. Two DUBs, USP15 and USP30, were shown to antagonize PARK2 activity during mitophagy (see sub-chapter 1.5.3 & 1.5.4) (Cornelissen et al., 2014; Cunningham et al., 2015). As I discussed in sub-chapter 1.5.3, USP8 is the only known DUB that promotes PARK2 activity during mitophagy (Durcan et al., 2014). Despite the intensified interest in mitochondrial DUBs, we have limited understanding in the role of these DUBs in healthy neurons under normal conditions. I will be discussing the potential roles of USP30 and USP35 in mitochondria under basal and mitophagy conditions.

### 5.3.1 USP30 may mediate mitochondrial morphology under basal conditions and delays PARK2 recruitment during mitophagy

USP30 was initially thought of as a “housekeeping” DUB that is involved in mitochondrial morphology regulation. When knocking down USP30 or inhibiting USP30 activity with a small molecule called “S3”, mitochondria become elongated and interconnected in both HeLa and MEF cells (Yue et al., 2014). Yue et al. reported that USP30 inhibition leads to an increase in ubiquitination of MFN2 under basal conditions. Importantly, S3-induced mitochondrial elongation is facilitated by MFN1 and MFN2, suggesting a relationship between USP30 and mammalian mitofusins (Yue et al., 2014). It has been reported that Fzo1p, the yeast ortholog of MFN1/2, requires ubiquitination and deubiquitination cycles in order to facilitate mitochondrial fusion (Anton et al., 2013). It is possible that MFN1/2 also require a similar mechanism to drive fusion and USP30 may regulate mitochondrial morphology by affecting MFN1/2 ubiquitination levels.

USP30 also has important functions on damaged mitochondria. I have demonstrated that knocking down USP30 led to an increase in PARK2-MFN2 interactions under basal conditions and during CCCP treatment (Fig. 3.12). I also observed that PARK2 recruitment time was delayed in USP30 overexpressing cells (Fig. 3.11). It has been proposed that PARK2 relies on PINK1-phosphorylated MFN2 to translocate to the mitochondria (Chen & Dorn II, 2013). A newly emerging hypothesis suggests that PARK2 utilizes a feedforward loop to translocate to the mitochondria. Upon mitophagy depolarization, only a few PARK2 are recruited to mitochondria and ubiquitinate OMM proteins. PINK1 phosphorylates ubiquitins and MFN2, which become the
main recruiters of PARK2 on the mitochondria. PARK2-mediated ubiquitination provides more substrates for PINK1 to phosphorylate, which in turn recruit more PARK2 (Ordureau et al., 2014). This positive feedback loop allows PARK2 to quickly accumulate on the surface of mitochondria. I propose a model in which USP30 regulates initial PARK2 recruitment time by interfering with the PARK2-MFN2 interaction, therefore delaying this positive feedback loop and giving the initially damaged mitochondria a chance to recover (Fig. 5.1).

5.3.1.1 Future Direction

The evidence supporting the existence of phosphorylated ubiquitin on the surface of mitochondria has reshaped the way we think about the mechanism of PARK2 recruitment and activation. We still do not know whether these phosphorylated ubiquitins can be cleaved by DUBs that antagonize PARK2 activity. Besides K48-linked and K63-linked ubiquitins, PARK2 also generates K6-linked and K11-linked ubiquitins. USP30 has been shown to preferentially cleave K6-, K11-, K48-, and K63-linked ubiquitin in vitro (Cunningham et al., 2015). Ordureau et al. proposed that USP30 does not prefer to cleave phosphorylated ubiquitin over unmodified ubiquitin (Ordureau et al., 2014). Therefore, USP30 specificity remains a debated topic and needs to be studied further.

Future works should include determining if the deubiquitinating activity of USP30 changes in the presence phosphorylated ubiquitin chains with different linkages. This can be tested by combining the in vitro deubiquitinating assays and phospho-tag gels. Purified linkage-specific di-UB can be subjected to in vitro phosphorylation (Kane et al., 2014). The phosphorylated ubiquitin can be then used to measure the deubiquitinating activity of purified USP30 in vitro (Cunningham et al., 2015). The cleavage of phosphorylated di-UB can be visualized by the changes in molecular weights of the ubiquitin bands on the phospho-tag gels. USP15 is another DUB that can antagonize PARK2 activity by deubiquitinating PARK2 substrates. Interestingly, USP15 is not localized to the mitochondria and does not physically interact with PARK2. Similar in vitro deubiquitinating assays can be done with USP15 to investigate its preference in ubiquitin linkages and phosphorylation status.
In healthy mitochondria (A), USP30 is involved in the regulation of mitochondrial fusion through the deubiquitination of MFN2. In mitochondria that are depolarized (B), PINK1 is stabilized on the OMM and phosphorylates MFN2. (C) Phosphorylated MFN2 serve as an initial signal to recruit PARK2 to the OMM, where PARK2 ubiquitinates OMM proteins, including MFN2. In the absence of USP30 (D), the initiation of PARK2 positive feedback loop is left unopposed. PARK2 ubiquitinates more MFN2 while PINK1 phosphorylates more ubiquitin. Phosphorylated ubiquitin recruits more PARK2 to the mitochondria. In the presence of USP30 (E), some PARK2-MFN2 interaction is disrupted. USP30 also deubiquitinates PARK2 substrates, delaying recruitment of PARK2 to OMM.

5.3.2 Possible roles of USP35 in mitochondrial quality control

What is the role of the USP35 complex under basal conditions? My data suggest that the USP35 complex may have a protective effect on mitochondrial quality under basal conditions. Even
though knocking down both s-USP35 and l-USP35 did not lead to obvious morphological changes in the mitochondria, it did lead to a drastic reduction in MFN2 both at the protein and mRNA levels (Fig. 3.8B). MFN2 is responsible for tethering mitochondria and ER, and knocking down MFN2 affects the Ca\textsuperscript{2+} transfer between the two organelles. Nie et al. also reported that knocking down MFN2 leads to increase in ROS production and decrease in mitochondrial potential (Nie et al., 2014). Therefore, MFN2 is essential to the health of the mitochondria and cell survival. Many environmental stimuli can alter the level of MFN2 mRNA. For example, the MFN2 mRNA level significantly decreases in neurons exposed to excitotoxicity as a result of a decrease in a transcription factor, MEF2. In cortical neurons expressing dominant negative MEF2, there was lower MFN2 promoter activity and mitochondrial potential in comparison to control cells, suggesting that MFN2 levels are regulated and linked to mitochondrial health. Both cytosolic and nuclear fractions of MEF2 can be ubiquitinated (Martorell-Riera et al, 2014).

It is possible that knocking down USP35 affects processes that regulate the mRNA level of MFN2. USP35 is present in both mitochondria and cytosol, which leads to two possible mechanisms to regulate MFN2 levels: 1) knocking down USP35 may directly affect mitochondrial health, leading to a signaling cascade that causes a decrease in MFN2 mRNA; 2) knocking down USP35 may affect the stability of a protein that controls MFN2 mRNA. Currently, little is known about the substrates and interacting partners of USP35, therefore it is difficult to predict how USP35 affects MFN2 and mitochondrial quality control.

Unlike USP30, USP35 did not play a strong antagonistic role against PARK2 during mitophagy. However, USP35 also interferes with PARK2-MFN2 interactions under basal conditions and during mitophagy (Fig. 3.12). Moreover, USP35 has the ability to delay PARK2-mediated mitochondrial clearance even when it is in the cytosol (Fig. 3.10A & B). Therefore, I proposed a model in which s-USP35 and l-USP35 form a stable complex within the cells. In healthy cells with polarized mitochondria, s-USP35 brings l-USP35 to the surface of the mitochondria, where l-USP35 interferes with PARK2-MFN2 interactions. USP35 also ensures that there is a sufficient amount of MFN2 through an unknown pathway. Upon mitochondrial depolarization, the USP35 complex dissociates from the mitochondria, allowing PARK2 to be recruited to the mitochondria and ubiquitinate OMM proteins. However, USP35 delays PARK2-mediated mitophagy from the cytosol through unclear mechanisms (Fig. 5.2).
### 5.3.2.1 Future Direction

Further work on identifying the substrates of USP35 is essential to understand how this complex works. Because DUBs have dynamic interactions with their substrates, conventional interaction identification methods, such as immunoprecipitation, may be too harsh to detect loosely bound substrates. The recent advances in stable isotope labeling by amino acids in cell culture (SILAC) methods and the commercialization of K-ε-GG antibody provide a platform for large-scale identification and quantification of the ubiquitinated substrates. SILAC is a quantification technique that allows one to compare the relative ratio of specific proteins between two treatments. Ubiquitinated substrates that are digested by trypsin leave a signature motif, K-ε-GG, which can be recognized by antibody (Cunningham et al., 2015). To identify potential USP35 substrates under basal conditions, one can perform SILAC on WT and USP35 knocked out cells. The ubiquitinated peptides can be isolated with K-ε-GG antibody and subjected to mass spectrometry. I hypothesize that USP35 may regulate the protein levels of transcription factors that up-regulates the transcription of MFN2, such as MEF2.

There are many questions remaining in the dynamics of the USP35 complex. First, we still don’t know how s-USP35 and l-USP35 interact with each other. I showed that s-USP35 and l-USP35 remain a complex under normal conditions and during mitochondrial damage. To determine where s-USP35 and l-USP35 bind, one can generate fragments of s-USP35 and l-USP35 and perform co-immunoprecipitation experiments. By identifying the binding sites, we will have a better understanding of how s-USP35 regulates the localization of l-USP35. Secondly, how s-USP35 dynamically associates and dissociates with the mitochondria remains unexplained. Because s-USP35 has the ability to dissociate from and re-associate with mitochondria, I hypothesized that s-USP35 is a peripheral protein that associates with OMM proteins. One possible way to screen for the mitochondrial binding partners of s-USP35 is to systematically knock down OMM proteins with RNAi and monitor the localization of s-USP35 with a high content screening system such as Opera Phire. If an siRNA induces s-USP35 to dissociate from the mitochondria, the targeted protein may be a candidate for binding partner. The interaction between s-USP35 and the potential candidate proteins can be validated by co-immunoprecipitation or fluorescence resonance energy transfer.
5.3.3 USP35 MAY REGULATE A MITOPHAGY SIGNALING HUB

In my thesis, I showed that while being in the cytosol, the USP35 complex delays PARK2-mediated mitochondrial clearance. This implies that USP35 may act through cytosolic pathways to antagonize PARK2 activity during mitophagy. The limited amount of literature on USP35 suggests that it may act as a tumor suppressor and regulate the NF-κB signaling pathway. Liu et al. showed that USP35 interacts with and stabilizes ABIN2, which is an inhibitor of NF-κB. Furthermore, USP35 overexpressing tumor exhibited delayed growth 6-weeks post xenograft in comparison to control tumor (Liu et al., 2015). The authors did not investigate in detail about the binding sites of USP35 and ABIN2.
ABIN1, ABIN2, OPTN, and NF-κB essential modulator (NEMO) share a highly conserved ubiquitin-binding domain, denoted NOAZ, which shows strong preference for binding to K63-linked ubiquitin. During mitochondrial stress, PARK2 promotes the linear ubiquitination of NEMO. NEMO is a part of a complex that phosphorylates IKB, an inhibitor of NF-κB, which leads to the ubiquitination and degradation of IKB. NF-κB transports into the nucleus and activates the transcription of targeted genes, one of the targeted genes activated by NF-κB is OPA1. These data suggest that there may be a signaling hub that regulates mitophagy, including NF-κB. NF-κB can upregulate the expression of BECN1 through a consensus promoter, therefore enhancing autophagy in vivo (Copetti et al., 2009). The NF-κB signaling pathway may be the missing link in the mechanism of how cytosolic USP35 delays mitophagy. USP35 may continue to delay PARK2-mediated mitophagy by suppressing NF-κB by stabilizing ABIN2 in the cytosol in order to counteract PARK2-mediated NF-κB activation (Fig. 5.3).

5.3.3.1 Future Direction

It is possible that during mitochondrial stress, s-USP35 and l-USP35 localize to the cytosol where the USP35 complex further delay mitophagy by inhibiting NF-κB signaling through the stabilization of ABIN2. To test if ABIN2 and NF-κB signaling is required to delay mitophagy, one can first overexpress ABIN2 or suppress ABIN2 expression and measure if there is a change in mitophagy rate in vivo. If NF-κB signals promotes mitophagy, knocking down ABIN2 would accelerate mitophagy while overexpressing ABIN2 would delay mitophagy. Furthermore, to test if the USP35-ABIN2 interaction is necessary for cytosolic the USP35 complex to delay mitophagy, one can express an ABIN2 mutant that does not bind USP35 and measure the rate of mitophagy. In addition, to test if USP35 translocation is important in NF-κB activation, one can anchor the USP35 complex on the mitochondria. By expressing an siRNA-resistant l-USP35 that is anchored on the OMM in a USP35<sup>−/−</sup> background, one can drain the cytosolic USP35 pool and measure NF-κB activation. It is also important to further study the role of NF-κB in PARK2-mediated mitophagy.
Figure 5.3. Proposed roles of PARK2 and the USP35 Complex in NF-κB signaling

Upon mitophagy activation, PARK2 activates NF-κB signaling by promoting NEMO linear ubiquitination. NF-κB travels into the nucleus and promotes the transcription of OPA1. The USP35 complex is localized to the cytosol, where the USP35 complex may deubiquitinate and stabilize ABIN2. ABIN2 may inhibit NF-κB signaling by inhibiting the nuclear translocation of NF-κB. The USP35 complex may still play an opposing role of PARK2 activity while it is in the cytosol.

5.4 CONCLUSION

Since the discovery of PARK2-mediated mitophagy, there has been tremendous interest in understanding how this process is implicated in Parkinson’s disease (PD). Although PARK2 is associated with early-onset familial Parkinsonism, which accounts for a small percentage of all PD cases, we believe that it can provide some information on the disease mechanism of PD. Currently there is no cure for PD, and the drugs provided for PD patients merely alleviate the
symptoms. Many patients eventually develop insensitivity to these drugs as their disease progresses. Looking for better drug targets has been a main driving force for the mitophagy field to progress.

It has become clear that many factors play an antagonistic role to PARK2 in mitophagy. This is not surprising because degrading healthy mitochondria unnecessarily is costly to the cells. However, these factors, such as DUBs, may do more harm than good in the context of PD by preventing PARK2 from quickly degrading damaged mitochondria. The key to identifying better drug targets may lie in these regulatory factors that are involved in mitophagy, not PARK2 itself. With the current interest in PD, I believe that more proteins will become available as suitable drug targets for PD and other neurodegenerative diseases.
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