Understanding the Role of the Co-Chaperone, Bcl-2 Associated Athanogene 5, in PINK1/parkin Dependent Mitophagy

by

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Abstract

Mutations in the genes encoding PINK1 and parkin are known to cause autosomal recessive familial Parkinson's disease (PD). PINK1 and parkin act in a sequential pathway to identify and tag damaged mitochondria for selective autophagic degradation, a process termed mitophagy. The co-chaperone protein, BAG5, has been shown to interact with both PINK1 and parkin, and to inhibit parkin's E3 ubiquitin ligase activity. Furthermore, BAG5 is known to enhance dopaminergic neurodegeneration in rodent models of PD. This thesis investigates the hypothesis that BAG5 negatively regulates PINK1 and parkin dependent mitophagy. We confirmed that BAG5 can interact with PINK1 and that this interaction requires functional BAG domains by co-immunoprecipitation. Fluorescence microscopy analysis revealed that BAG5 impairs GFP-parkin recruitment to depolarized mitochondria. This in turn impaired downstream mitophagy, as measured by a flow cytometry assay directly quantifying lysosomal engulfment of mitochondria. These findings demonstrate a potential role BAG5 in PD pathobiology.

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This thesis is dedicated to my father, whose own ordeal with Parkinson's disease has inspired me to pursue research in this field.

Contributions

Victoria Agapova – Performed blinded counts of cells displaying GFP-parkin translocation to mitochondria that are quantified in Figure 7.

Dr. Edward Fon and Dr. Matthew Tang – Created and/or provided each of the various U2OS stable cell lines used through this thesis.

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List of Abbreviations

BAG = Bcl-2 associated athanogene
BNB = Brand new BAG
CCCP = Carbonyl cyanide *m*-chlorophenyl hydrazone
CHIP = C-terminal of Hsp70 interacting protein
CMA = Chaperone mediated autophagy
DBS = Deep brain stimulation
DUB = Deubiquitinating enzyme
FBS = Fetal bovine serum
GPi = Globus pallidus internus
GST = Glutathione S-transferase
Hsc = Heat shock cognate
Hsp = Heat shock protein
LC3 = Microtubule-associated protein 1A/1B-light chain 3
LRRK2 = Leucine-rich repeat kinase 2
MDV = Mitochondrial derived vesicle
MFB = Medial forebrain axotomy
MOA = Monoamine oxidase
MPP = mitochondrial processing peptidase
PTEN = Phosphatase and tensin homolog
PVDF = Polyvinylidene fluoride
RBR = Ring-between-ring
RING = Really interesting new gene
RIPA = Radioimmunoprecipitation assay
RNAi = RNA interference
SBD = Substrate binding domain
SD = Standard deviation
SEM = Standard error of the mean
MPP+ = 1-methyl-4-phenylpyridinium ion
MPPP = 1-methyl-4-phenyl-4-propionoxypiperidine
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTS = Mitochondrial targeting sequence
NA = Numerical aperture
NBD = Nucleotide binding domain
NBR1 = Neighbor of BRCA1 gene 1
NDP52 = Nuclear dot protein 52
Nrf-1 = Nuclear respiratory factor 1
OMM = Outer mitochondrial membrane
OPTN = optineurin
p62 = Nucleoporin 62
PARIS = Parkin interacting substrate
PARL = Presenilin-associated rhomboid-like protein
PBS = Phosphate buffered saline
PD = Parkinson's disease
PINK1 = PTEN-induced putative kinase 1

siRNA = short interfering RNA
SN = Substantia nigra
SNpc = Substantia nigra pars compacta
SNpr = Substantia nigra pas reticula
TBS = Tris buffered saline
TBST = TBS-Tween 20
Ubl = Ubiquitin-like domain
UPS = Ubiquitin proteasome system
$\Delta\psi m$ = Mitochondrial membrane potential

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Introduction

0.1 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting approximately 1-2% of the population over the age of 65 (Pringsheim et al., 2014). It was first described in 1817 by Dr. James Parkinson, the British physician from whom the disease derives its name, in his seminal work "An Essay on the Shaking Palsy". He documented the cardinal motor features of the disease including tremor, rigidity, akinesia, and postural instability (Parkinson, 1817). Today, it is recognized that patients often also suffer from debilitating non-motor symptoms such as olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, urinary incontinence, constipation, pain, and fatigue (Kalia and Lang, 2015). Both diagnosis and treatment of the disease are limited; Diagnoses of PD are made purely on clinical criteria, as there is currently no objective biomarker, and existing treatments are only able to target symptoms and lack disease modifying properties.

Pathologically, PD is characterized by the selective and progressive loss of the dopaminergic neurons of the substantia nigra pars (SNpc) and the presence of Lewy pathology in remaining neurons. Lewy bodies and neurites are insoluble proteinaceous inclusions primarily composed of misfolded α-synuclein found in the neural cell body and processes, respectively. SNpc degeneration is extensive with symptoms only becoming apparent after 60-70% loss of dopaminergic neurons. The main target of the SNpc dopaminergic terminals is the striatum and the loss of dopamine tone here results in dysfunction of the basal ganglia, a subcortical circuit responsible for fine tuning of motor control. It is this dysregulation of the excitatory and inhibitory connections in the striatum that account for the hypokinesia seen in PD.

Current gold standard treatments are directed at correcting this dysfunction of the basal ganglia. Dopamine replacement is achieved pharmacologically through the administration of the dopamine precursor L-dopa, or other dopamine agonists. While highly effective at managing symptoms early in disease progression, L-dopa treatment becomes less effective over time and is associated with debilitating side effects including motor fluctuations, impulse control disorders, and hallucinations. Motor fluctuations, also referred to as ON-OFF phenomenon, occur when brain dopamine levels are closely tied to plasma L-dopa levels and high levels following administration result in dyskinesia, while low levels cause the patient to return to a hypokinetic state. Surgically, deep brain stimulation (DBS) of the subthalamic nucleus or the globus pallidus internus (GPi) directly modifies the electrical activity of the circuit. DBS is effective in managing the motor symptoms of PD, but it is reserved for a subset of patients that respond well to L-dopa but experience disabling side effects, and do not have cognitive impairment.

The etiology of PD is complex and unclear with both genetics and environmental factors playing a role. Evidence that has emerged from case study, epidemiological, and genetic studies points to a central role for mitochondrial dysfunction and disturbed proteostasis in PD pathogenesis.

0.2 Mitochondrial Dysfunction in Parkinson's Disease

Some of the first evidence linking mitochondrial dysfunction to PD emerged from a case study documenting several individuals in Northern California who developed severe parkinsonism within a week of accidentally injecting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a byproduct in the synthesis of the "synthetic heroin", 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) (Langston et al., 1983). MPTP exposure faithfully and severely recreated the cardinal motor symptoms of PD through the rapid and selective degeneration of the SN (Langston et al., 1983). The mechanism of this selective degeneration was found to depend on the conversion of

MPTP to its toxic metabolite, 1-methyl-4-phenylpyridinium ion (MPP+), by monoamine oxidase B (MOA-B) (Heikkila et al., 1984). This conversion occurs in astrocytes (Ransom et al., 1987) and serotonergic neurons (Shen et al., 1985) after the lipophilic MPTP has crossed the blood brain barrier. The selectivity for dopaminergic neurons in conferred by uptake through the dopamine transporter (DAT) (Shen et al., 1985), and uptake is greater in SN dopaminergic neurons than the dopaminergic neurons of the ventral tegmental nucleus due to higher concentrations of DAT (Kitayama et al., 1993). Once inside the neuron, MPP+ accumulates in the mitochondria where it inhibits Complex I of the mitochondrial respiratory chain (Ramsay et al., 1986) causing a decrease in ATP (Chan et al., 1991) and potentially increased reactive oxygen species (ROS).

MPTP has been used to create the majority of non-human primate models that have been employed in the development of PD therapeutics, although they have been criticized for not replicating the chronic nature and Lewy pathology seen in PD (Fox and Brotchie, 2010). Interestingly, rodents, especially rats, have a marked resistance to systemic MPTP treatment because of high concentrations of MOA in their blood brain barrier which converts MPTP to its electrically charged metabolite, MPP+, which prevents it from crossing (Riachi et al., 1988). In mice, who have an intermediate susceptibility to MPTP, α-synuclein knockout is shown to confer resistance to MPTP administration (Dauer et al., 2002), providing a potential link between environmental and genetic PD factors. Additional rodent models also implicate mitochondrial dysfunction in PD as parkinsonism can also be induced in rats through the systemic administration of rotenone (Betarbet et al., 2000) and paraquat (Cicchetti et al., 2005), two pesticides and mitochondrial toxins that inhibit Complex I and generate ROS, respectively.

Informed by the specific inhibition of Complex I by MPTP, Schapira et al. compared the activity of electron transport chain enzymes from the SN of PD patients to age matched controls. They found deficiencies in the Complex I enzymes, NADH cytochrome *c* reductase and NADH CoQ reductase, but not in Complex II or III enzymes, in the samples from PD patients (Schapira et al., 1990). Moreover, PD patients are known to accumulate mitochondrial DNA (mtDNA) mutations (Bender et al., 2006; Lin et al., 2012). Epidemiological evidence also supports a role for mitochondrial dysfunction in PD as population studies indicate that both paraquat and rotenone exposure are associated with an increased risk of developing PD (Noyce et al., 2012; Tanner et al., 2011). Several clinical trials have attempted to modify PD progression by administering antioxidants or bioenergetics aimed at improving mitochondrial function. These include trials of vitamin E (The Parkinson Study Group, 1989), mitoquinone (Snow et al., 2010), Coenzyme Q10 (The Parkinson Study Group, 2014), and creatine (Kieburtz et al., 2015). These trials all showed no significant difference between treatment groups at their endpoints, but these failures may be the result of initiating trials too late in the disease progression, or failing to select a clinical subpopulation that might benefit the most (Espay et al., 2017).

Later discoveries of monogenic forms of PD provide further support for the hypothesis that mitochondrial dysfunction plays a central role in PD pathogenesis, while also implicating other key molecular pathways.

0.3 Genes that Cause Parkinson's Disease

The vast majority of PD cases are sporadic in nature, but monogenic forms of the disease account for approximately 5-10% of cases (Lesage and Brice, 2009). Identification of these genes and the proteins that they encode has provided tremendous insight into the biological mechanisms underpinning PD pathobiology. There are currently five genes known to cause PD

that have been well replicated. These include mutations in two genes that cause autosomal dominant PD, *SCNA (PARK1/PARK4)* and *LRRK2 (PARK8),* and three genes that cause autosomal recessive PD, *parkin (PARK2), PINK1 (PARK6),* and *DJ-1 (PARK7).* A full collection of the mutations in these genes published to date can be found in the Parkinson Disease Mutation database (PDmutDB, http://www.molgen.vib-ua.be/PDmutDB). The proteins encoded by the three recessive genes (*parkin, PINK1,* and *DJ-1*) are known to directly affect mitochondrial function and health, and there is evidence that *SNCA* impairs mitochondrial function.

In 1997, *SNCA,* the gene encoding α-synuclein, was the first gene identified to cause PD; Italian and Greek families were found to have a pathogenic missense mutation resulting in an alanine to threonine substitution at amino acid 53 (Polymeropoulos et al., 1997). Two other point mutations, A30P (Kruger et al., 1998) and E46K (Zarranz et al., 2004), as well as triplication (Singleton et al., 2003) and duplication (Ibanez et al., 2004) of the *SNCA* locus have since been shown to cause PD. Only months following the discovery of the A53T pathological mutant, insoluble α-synuclein was found to makeup Lewy bodies (Spillantini et al., 1997). While Lewy bodies were originally postulated to be the neurotoxic form of α-synuclein, cases of PD lacking Lewy pathology and the evidence of different types α-synuclein aggregation have implicated certain soluble α-synuclein oligomers as the toxic species (Kalia et al., 2013).

While the mechanisms by which oligomeric α-synuclein species cause neurodegeneration are still being elucidated, multiple lines of evidence indicate that it accumulates in mitochondria and impairs Complex I of the mitochondrial respiratory chain (Chinta et al., 2010; Devi et al., 2008; Liu et al., 2009; Luth et al., 2014). Oligomeric and dopamine-modified species of α-synuclein have also been shown to interact the translocase of the outer mitochondrial membrane (TOMM) subunit, TOMM20, blocking mitochondrial protein import and impairing mitochondrial

respiration in rodent PD models (Di Maio et al., 2016). This group also showed that this α-synuclein-TOMM20 interaction, and impaired mitochondrial import were present in SN dopaminergic neurons from human PD brains, but not healthy controls (Di Maio et al., 2016). Monomeric α-synuclein may also play a physiological role in mitochondrial function, as α-, β-, and γ-synuclein triple knockout mice show deficiencies in mitochondrial respiration and ATP synthase efficiency that can be rescued by the addition of low levels of monomeric α-synuclein (Ludtmann et al., 2016).

Mutations in *LRRK2,* the gene encoding leucine-rich repeat kinase 2 (LRRK2), are found in 4% of familial and 1% of sporadic PD, making them the most common cause of genetic PD (Healy et al., 2008). The most common LRRK2 mutation, G2019S, causes a toxic enhancement of kinase function (Smith et al., 2006). The clinical phenotype seen in patients harbouring *LRRK2* mutations, including the age of onset, is like that of classical PD. The neuropathology, however, is heterogenous and can present as typical LB pathology, Alzheimer's disease-like tau positive pathology without LBs, or nigral degeneration without LB or tau pathology (Nuytemans et al., 2010).

Deletions and loss of function mutations in the Daisuke-Junko-1 (*DJ-1)* gene result in early-onset parkinsonism (Annesi et al., 2005; Bonifati et al., 2003). While the function of DJ-1 is not well understood, the protein localizes to the mitochondria (Zhang et al., 2005) and is believed to protect cells from oxidative stress (Guzman et al., 2010; Kim et al., 2005; Yokota et al., 2003).

PINK1 and *PARK2* are the most well characterized of the autosomal recessive PD genes. Their structure and function, particularly in directing mitophagy, the selective autophagic degradation of mitochondria, is discussed in detail below.

0.4 PINK1 and Parkin

0.4.1 Domains and Structure

Phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) is a 581-amino acid, 62 kDa, serine threonine kinase (Valente et al., 2004). PINK1 has a N-terminal mitochondrial targeting sequence (MTS), a transmembrane domain, a large serine threonine kinase domain, and a conserved C-terminal region (CTR) (Sim et al., 2012) (Figure 1). Pathological mutations include missense mutations in all of these domains, nonsense mutations, and exon rearrangements, with missense and nonsense mutations being the most common (Kalia and Lang, 2015). Clinically, patients with *PINK1* mutations present with early-onset parkinsonism that is otherwise indistinguishable from idiopathic PD (Nuytemans et al., 2010). Heterozygous mutations in *PINK1* are also a risk factor for PD (Puschmann et al., 2017).

The crystal structure of human PINK1 has not been solved, but recent reports have described the crystal structure of PINK1 from the insect species, *Tribolium castaneum* (TcPINK1) (Kumar et al., 2017), as well as the structure of *Pediculus humanus corporis* PINK1 (PhPINK1) in complex with its substrate, ubiquitin (Schubert et al., 2017). Insect PINK1 orthologues are commonly used to study PINK1 *in vitro* because they share great sequence homology to human PINK1, and unlike recombinant human PINK1, they can be efficiently produced and display substantial kinase activity *in vitro* (Woodroof et al., 2011). The N-lobe of the PINK1 kinase domain contains three insertions and the structures of TcPINK1 and PhPINK1 both indicate that ubiquitin binding occurs on the highly conversed third insertion (Kumar et al., 2017; Schubert et al., 2017). The ATP-binding pocket is located in the C-lobe of the kinase domain, and the CTR is important for PINK1 stability because it contains four α-helices that pack tightly around the C-lobe forming an extended hydrophobic core (Schubert et al., 2017). PINK1 is regulated by

(auto)phosphorylation at two sites, Ser202 and Ser204 in PhPINK1 and Ser228 and Ser230 in human PINK1, that are necessary for the correct positioning of N-lobe insertion 3 and insertion 2, respectively (Schubert et al., 2017). Interestingly, PINK1 appears to prefer to phosphorylate ubiquitin that is in an uncommon C-terminally retracted conformation (Gladkova et al., 2017).

Parkin is a 465 amino acid, 52 kDa, really interesting new gene (RING)-in-between-RING (RBR) E3 ubiquitin ligase (Shimura et al., 2000). Conserved domains include an N-terminal ubiquitin-like (Ubl) domain and two C-terminal RING domains, RING1 and RING2, that are joined by an in-between RING (IBR) domain and a unique linker termed the repressor element of parkin (REP) (Trempe et al., 2013) (Figure 1). Compared to other RBR E3 ligases, parkin has a unique third zinc finger, RING0, immediately to the N-terminal side of RING1 (Hristova et al., 2009). Parkin, like other RBR E3s, uses a RING/HECT hybrid mechanism where its RING1 domain binds with E2 ubiquitin conjugating (Ubc) enzymes and it transfers ubiquitin to its substrates through a high-energy thioester intermediate formed on conserved cysteine 431 in its RING2 domain (Wenzel et al., 2011). Like PINK1, pathogenic parkin mutations are found in all domains and linker regions (Ferreira and Massano, 2017). Parkin mutations are the most common cause of early-onset parkinsonism, accounting for 50% of familial and 15% of sporadic cases that have an age of onset before 45 (Lücking et al., 2000; Periquet et al., 2003). Interesting, a majority of parkin mutant PD cases lack Lewy pathology and cognitive impairment, and are associated with slow disease progression (Doherty et al., 2013).

Parkin exists in an autoinhibited state; the crystal structure of parkin shows that this state is maintained by a rigid core that is stabilized by hydrophobic interactions between RING0, RING1, and RING2 (Trempe et al., 2013). Three key interactions mediate this autoinhibition. Interaction between the Ubl and RING1 blocks phosphorylation of the activating Ser65 residue

(Trempe et al., 2013). Binding of the REP to RING1 blocks the E2 interaction site on RING1 (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). Lastly, RING0 binding to RING2 blocks the catalytic C431 (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). Mutations designed to disrupt any of these interactions activate parkin and enhance its function in mitophagy (Tang et al., 2017). These interactions are relieved by binding to phospho-ubiquitin, a parkin receptor that activates parkin by inducing a conformational changes to expose the Ubl for phosphorylation, and places the E2 binding site and Cys431 in close proximity (Okatsu et al., 2015; Wauer et al., 2015). Release of the REP:RING1 and RING0:RING2 binding are more important than Ubl:RING1 in activating parkin (Tang et al., 2017). The sequential activation of PINK1 and parkin by mitochondrial stressors including mitochondrial depolarization by pharmacological agents and reactive oxygen species, or misfolded protein accumulation triggers mitophagy (Pickrell and Youle, 2015).

FIGURE 1

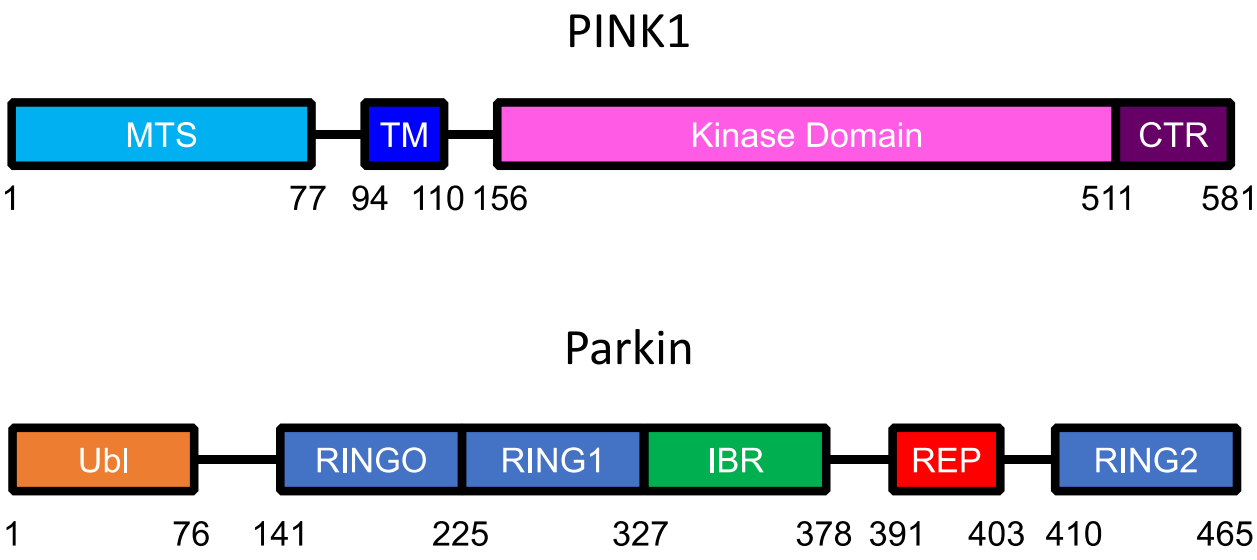

Figure 1: Schematic illustrations of PINK1 and parkin primary structure and domains.

0.4.2 PINK1 and Parkin Dependent Mitophagy

Canonical PINK1/parkin directed mitophagy relies on PINK1 to sense damaged mitochondria and activate parkin, which then decorates the mitochondria with polyubiquitin chains. In healthy mitochondria, PINK1 is constitutively imported through the TOMM complex into the translocase of inner mitochondrial membrane (TIMM) where its mitochondrial targeting sequence is first cleaved in the matrix by mitochondrial processing peptidase (MPP) (Greene et al., 2012). PINK1 is further cleaved in its transmembrane domain by the inner membrane protein rhomboid protease, presenilin-associated rhomboid-like protein (PARL) (Jin et al., 2010), generating a 52 kDa fragment with an N-terminal phenylalanine that is rapidly degraded by the proteasome according to the N-end rule (Deas et al., 2011; Yamano and Youle, 2013). Dissipation of mitochondrial membrane potential, however, prevents this cleavage by blocking import into the TIMM complex, leading to PINK1 accumulation of the mitochondrial surface (Matsuda et al., 2010; Narendra et al., 2010b). The small TOMM subunit, TOMM7, is required for this accumulation (Hasson et al., 2013). PINK1 can also be stabilized on the surface of polarized mitochondria in response to abnormal accumulations of misfolded proteins in the mitochondrial matrix (Jin and Youle, 2013). While the mechanism of PINK1 stabilization on polarized mitochondria is not fully understood, the authors of this study believe that the accumulation of misfolded protein in the mitochondria also blocks import by TIMM complex (Jin and Youle, 2013).

Stabilization on the mitochondrial membrane allows PINK1 to perform is serine/threonine kinase function resulting in parkin activation and recruitment from the cytosol to the mitochondrial surface (Matsuda et al., 2010; Narendra et al., 2010b). This is achieved through phosphorylation of parkin’s ubiquitin-like domain (Ubl) and more importantly, ubiquitin at serine 65 by PINK1

(Kane et al., 2014; Kondapalli et al., 2012; Koyano et al., 2014). Upon activation, parkin ubiquitinates itself and several outer mitochondrial membrane (OMM) proteins leading to their degradation by the ubiquitin-proteasome system, and autophagosome engulfment of the mitochondria (Chan et al., 2011; Narendra et al., 2008; Sarraf et al., 2013).

Several proteins that modify the PINK1/parkin pathway have been identified. Genome wide RNA interference (RNAi) screens have identified ATPase inhibitory factor 1 (ATPIF1) (Lefebvre et al., 2013), hexokinases 1 and 2 (McCoy et al., 2014), HSPA1L (Hasson et al., 2013), and sterol regulatory element binding transcription factor (SREBF) (Ivatt et al., 2014), as proteins that when knocked down reduce parkin translocation to mitochondria, indicating that they normally function to support parkin recruitment. Conversely, other proteins have been identified that when knocked down, enhance parkin translocation to mitochondria, indicating that they normally inhibit this process. Some of these proteins include, Bcl-2 associated athanogene 4 (BAG4) and seven in absentia homolog 3 (SIAH3) (Hasson et al., 2013), and the deubiquitinating enzyme (DUB) USP30 (Bingol et al., 2014; Wang et al., 2015). While these USP30 antagonizes parkin mediated mitophagy by removing ubiquitin chains from mitochondria, another DUB, USP8, interestingly accelerates parkin recruitment and mitophagy by removing K6 linked ubiquitin chains from parkin itself (Durcan et al., 2014). Two more DUBs, USP15 and USP35, impair parkin mediated mitophagy without affecting parkin recruitment to the mitochondria (Cornelissen et al., 2014; Wang et al., 2015).

Besides Jin and Youle (2013), who used the expression of a misfolded mitochondrial protein, the vast majority of studies examining mitophagy in cells have employed pharmacological treatment with ionophores like carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and valinomycin, or combinations of the respiratory chain inhibitors, oligomycin and antimycin A, to model

mitochondrial dysfunction. This approach leads to complete damage and clearance of whole mitochondrial networks. A recent study, however, has demonstrated that PINK1 and parkin can direct the selective excision and mitophagy of mitochondrial subdomains containing aggregated proteins from the remaining healthy mitochondria (Burman et al., 2017). This selectively was found to depend on the function of mitochondrial fission promoting, dynamin-related protein 1 (Drp1), as Drp1 knockout cells could not separate the aggregate containing mitochondrial subdomains and instead underwent wholesale mitophagy (Burman et al., 2017).

Both PINK1 and parkin were originally believed to be necessary for mitophagy, however, PINK1, albeit with decreased efficiency, has been shown to be able to initiate mitophagy in the absence of parkin (Lazarou et al., 2015). This finding implies that ubiquitin phosphorylation by PINK1 is the key step in inducing mitophagy, and parkin amplifies this feedforward cycle by providing more substrate. This phospho-ubiquitin signal on mitochondria recruits specific autophagy receptors, proteins that link the cargo tagged for degradation to the autophagosome through interactions with both the specific degradation signals on the cargo, and lipidated microtubule-associated protein 1A/1B-light chain 3 (LC3) on the autophagosome (Stolz et al., 2014). The autophagy receptors, optineurin (OPTN) and nuclear dot protein 52 (NDP52), but not neighbor of BRCA1 gene 1 (NBR1) and nucleoporin 62 (p62), operate redundantly in mitophagy (Lazarou et al., 2015). p62 is, however, required for perinuclear clustering of mitochondria that typically precedes mitophagy (Narendra et al., 2010a; Okatsu et al., 2010).

While some early reports evaluating PINK1/parkin function in primary neuronal cultures found that neurons' reliance on oxidative phosphorylation prevented them from undergoing mitophagy (Van Laar et al., 2011), later studies showed that neurons do indeed perform PINK1/parkin dependent mitophagy (Ashrafi et al., 2014; Cai et al., 2012; Seibler et al., 2011). Neurons also

degrade mitochondria in a PINK1/parkin dependent manner at baseline, unperturbed conditions (Bingol et al., 2014).

0.4.3 PINK1 and Parkin *In Vivo*

The first evidence that parkin was important for maintaining healthy mitochondria came from parkin null *Drosophila* that showed pathological phenotypes characterized by reduced lifespan, degeneration of flight muscles, and male sterility with underlying mitochondrial pathology (Greene et al., 2003). Further studies in *Drosophila* provided the first evidence that PINK1 acted upstream of parkin in the same pathway as PINK1 null flies displayed the same phenotype as parkin null mutants and overexpression of parkin could overcome PINK1 deficit, but not vice versa (Clark et al., 2006; Park et al., 2006). Using radioisotope labeling in *Drosophila* brains followed by mass spectroscopy, Vincow et al. (2013) showed that parkin null flies had less turnover of mitochondrial proteins compared to wild-type flies and that both PINK1 and parkin null flies had specific decreases in the turnover of mitochondrial respiratory chain proteins, providing direct evidence for mitophagy occurring *in vivo.*

Unlike humans and flies, neither single nor double germline knockout of PINK1 and parkin result in severe motor or neuropathological phenotypes in mice (Goldberg et al., 2003; Kitada et al., 2007; Kitada et al., 2009; Perez and Palmiter, 2005). Resistance to loss of these genes could be conferred by abnormal compensation methods and indeed post-natal conditional knockouts of parkin have shown nigrostriatal degeneration (Shin et al., 2011; Stevens et al., 2015). Furthermore, stressing parkin knockout mice with a proofreading deficiency in mtDNA polymerase γ causes an excessive accumulation of mtDNA mutations that leads to specific degeneration of dopaminergic neurons, indicating a role for parkin mitochondrial quality control in mice (Pickrell et al., 2015). While initial mice studies complicated the role of PINK1 and

parkin, substantial evidence from flies and humans supports a model that PINK1 and parkin are essential for mitochondrial quality control *in vivo.*

0.4.4 Functions Outside of Mitophagy

PINK1 and parkin participate in several functions outside of wholesale mitophagy, including mitochondrial derived vesicle (MDV) formation, mitochondria biogenesis, and cell death. PINK1 and parkin can initiate the piecewise degradation of mitochondrial components through the generation of MDVs containing oxidized proteins (McLelland et al., 2014). These MDVs require syntaxin-17 but not Drp1 for delivery of their cargo to the endolysosomal system (McLelland et al., 2016). This makes them distinct from the mitophagy of selective mitochondrial fragments that do require Drp1 but not syntaxin-17 for degradation (Burman et al., 2017). MDV formation and degradation of specific mitochondrial proteins may represent a more physiological response to less harsh mitochondrial insult (McLelland et al., 2014).

In addition to its mitochondrial targets, parkin activation also leads to the ubiquitination of several cytosolic substrates (Sarraf et al., 2013). One of these substrates, parkin interacting substrate (PARIS), represses transcription of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis, which is decreased in SN from human PD patients compared to healthy controls (Shin et al., 2011). Parkin promotes mitochondrial biogenesis by ubiquitinating PARIS for degradation by the ubiquitin proteasome system (UPS), allowing for expression of PGC-1α target genes including nuclear respiratory factor 1 (Nrf-1) (Shin et al., 2011). PARIS accumulation seems to be responsible for the specific dopaminergic neurodegeneration seen in conditional parkin knockout mice as overexpression of PGC-1α is protective and prevents deficiencies in mitochondrial respiration (Lee et al., 2017; Shin et al., 2011; Stevens et al., 2015). PINK1 also plays a role in regulating

PARIS accumulation by phosphorylating PARIS to control ubiquitination and clearance by parkin (Lee et al., 2017).

The general neuroprotective effect of parkin has led to the hypothesis that it is always stress protective. Indeed, parkin has been shown to confer apoptosis resistance to a number of cellular stressors by enhancing ubiquitination of NF-κB essential modulator (NEMO) (Müller-Rischart et al., 2013). Furthermore, parkin has been shown to be protect cells against etoposide and staurosporine-induced apoptosis by regulating the proapoptotic, Bcl-2-associated X protein (BAX) (Charan et al., 2014; Johnson et al., 2012). Other groups, however, have shown that PINK1 and parkin actually enhance apoptosis in response to mitochondrial depolarization by ubiquitinating the antiapoptotic Bcl-2 family member, Mcl-1 (Carroll et al., 2014; Zhang et al., 2014). Together these results suggest that parkin may play a context dependent role in moderating cell death.

0.5 Chaperones

Chaperone proteins are the highly conserved molecular machinery responsibly for directing the folding of nascent polypeptide chains, refolding misfolded proteins, and targeting misfolded proteins for degradation. These proteins were initially found to be upregulated in the cellular stress response to elevated temperatures and were therefore termed heat shock proteins (Hsp) (Tissiéres et al., 1974). Constitutively expressed homologs of these inducible proteins are referred to as heat shock cognates (Hsc). Chaperone families were initially classified based on their molecular weights and include the Hsp40, Hsp60, Hsp70 Hsp90, Hsp100, and the small Hsp families. The growing number and heterogeneity of known chaperone proteins has necessitated a revised nomenclature system where DNAJ, HSPD, HSPA, HSPC, HSPH, and HSPB replace the respective molecular weight derived names (Kampinga et al., 2009). In this

thesis, the more common, older Hsp(kDA) notation will be used when referring to whole chaperone families, and the new nomenclature will be used when referring to specific chaperones.

The Hsp70s are the most well studied family of chaperones. Hsp70 accomplishes its protein refolding function by binding to exposed hydrophobic domains on misfolded proteins via its C-terminal substrate binding domain (SBD) and undergoing a multiple ATP hydrolysis cycles at its N-terminal nucleotide binding domain (NBD) (Bukau and Horwich, 1998). Hydrolysis of ATP to ADP stabilizes Hsp70's hold on its client protein, increasing the likelihood of spontaneous refolding (Ciechanover and Kwon, 2017). ADP-ATP exchange destabilizes the Hsp70-client complex, allowing for dissociation of the client or further ATP hydrolysis cycles (Friesen et al., 2017). Chaperone function in targeting misfolded proteins for degradation is achieved through interactions with non-client binding proteins, known as co-chaperones. C-terminal of Hsp70 interacting protein (CHIP), is an Hsp70 co-chaperone and E3 ubiquitin ligase that targets unfolded proteins for degradation by the UPS (Meacham et al., 2001; Murata et al., 2001). HSPA8, working with lysosomal-associated membrane protein 2A (LAMP2A) and multiple co-chaperones, can also identify substrates with a KFERQ-like motif for chaperone-mediated autophagy (CMA), a process that leads to the lysosomal degradation of specific proteins (Kaushik and Cuervo, 2012). The central nature of disturbed proteostasis and α-synuclein aggregation in PD has prompted great interest into the role of chaperones in PD pathogenesis, and their potential as therapeutic targets, topics reviewed in detail by my fellow graduate students and myself (Friesen et al., 2017).

0.5.1 Role in Parkinson's Disease

Multiple chaperone proteins have been found to accumulate in Lewy bodies (Sharma et al., 2001; Shashidharan et al., 2000). Studies in *Drosphilia* highlight a neuroprotective role for Hsp70 in PD as its overexpression attenuates the dopaminergic neurodegeneration caused by α-synuclein overexpression (Auluck et al., 2002). Building on this finding, other groups have shown that the overexpression of several Hsp70 and Hsp40 family members antagonize α-synuclein aggregation and/or toxicity in yeast (Flower et al., 2005), cells *(*McLean et al., 2002), and rodents (Klucken et al., 2004b; Moloney et al., 2014). The mechanisms by which Hsp70 attenuates α-synuclein aggregation and toxicity appear to be separate, as a mutation (K71S) that disrupts the NBD of Hsp70 and abolishes its refolding activity fails to protect cells from α-synuclein toxicity but does still prevent aggregation (Klucken et al., 2004a).

Hsp70 interactions with its co-chaperones can also attenuate α-synuclein toxicity through CMA and UPS degradation of aggregates. Expression of HSPA8 and lysosome-associated membrane protein 2 A (LAMP2A) are elevated in response to α-synuclein overexpression, indicating that they normally function to clear aggregates (Mak et al., 2010). Indeed, overexpression of LAMP2A, the rate-limiting component of CMA, protected rats from α-synuclein induced neurodegeneration (Xilouri et al., 2013). Mutant A53T and A30P α-synuclein, however, block CMA and inhibit their own degradation as well as degradation of other substrates by preventing their uptake into the lysosome (Cuervo et al., 2004). The co-chaperone, CHIP, can target *α*-synuclein for degradation by either the proteasome or lysosome via its TPR domain or U-box domain, respectively (Shin et al., 2005). CHIP also suppresses the formation of toxic α-synuclein oligomers by ubiquitinating them for degradation by the UPS (Kalia et al., 2011). However, this

CHIP-mediated suppression of α-synuclein oligomer formation is antagonized by another co-chaperone, BAG5 (Kalia et al., 2011).

The thesis is primarily concerned with BAG5 functions outside of its role as a co-chaperone, specifically in modulating PINK1 and parkin dependent mitophagy.

0.6 BAG5

The Bcl-2 associated athanogene (BAG) family is a diverse group of Hsp70 interacting co-chaperone proteins named for its founding member, BAG1. BAG1 was first identified as a Bcl-2 interactor in a yeast two-hybrid screen and was subsequently found to confer resistance to cell death elicited by apoptosis inducers (Takayama et al., 1995). Further yeast two-hybrid screens by the same group using the NBD of Hsp70 as bait identified BAG2 and BAG3 (Takayama et al., 1999). Sequence homology between the BAG domains, the family's characteristic C-terminal region that mediates their interactions with Hsp70, of BAG3 and the then unidentified, BAG5, led to BAG5's tentative classification as a BAG family member (Takayama et al., 1999). The BAG domain is made of a three parallel α-helices, where helices 2 and 3 mediate the interaction with the Hsp70 NBD (Sondermann et al., 2001). Compared to BAG1, the BAG domains of BAG3, BAG4, and BAG5 are shorter with each α-helix featuring three to four fewer turns (Briknarová et al., 2002). In BAG domain of BAG2, the three α-helices are not all parallel and actually bind Hsp70 differently, leading some to classify it as its own unique domain termed the brand new bag (BNB) domain (Xu et al., 2008). BAG5 is the only BAG protein to contain multiple BAG domains, five in total (Arakawa et al., 2010). Following its classification as a BAG family member by Takayama et al. (1999), BAG5 remained uncharacterized until it was implicated in neurodegeneration.

0.6.1 Role in Neurodegeneration

A role for BAG5 in neurodegeneration was initially hypothesized after it was identified as a differentially expressed transcript in the medial forebrain (MFB) axotomy rodent model of PD (Kalia et al., 2004). Its overexpression was subsequently shown to exacerbate neurodegeneration in that same model, as well as the MPTP model of PD (Kalia et al., 2004). BAG5 is highly expressed in both rat and human brain, and co-localizes with Lewy bodies in diseased human brain (Kalia et al., 2004). Moreover, BAG5 is also known to interact directly with a number of parkinsonian gene products including LRRK2 (Beilina et al., 2014), DJ-1 (Qin et al., 2017), parkin (Kalia et al., 2004), and PINK1 (Wang et al., 2014), as well as indirectly modulate α-synuclein (Kalia et al., 2011) (Figure 2).

As part of a large protein complex also including cyclin-G–associated kinase (GAK) and RAB7, member RAS oncogene family-like 1 (RAB7L1), BAG5's interaction with LRRK2 serves to promote the clearance of the trans-Golgi network in cells and *in vivo* (Beilina et al., 2014). This enhanced clearance leads to a reduction in neurite lengths in primary neuronal cultures (Beilina et al., 2014). In cell models of oxidative stress, BAG5 has recently been shown to attenuate DJ-1's protective effect on cell death by impairing its dimerization and recruitment to mitochondria (Qin et al., 2017).

The potential for BAG5 participation in PINK1 and parkin dependent mitophagy is highlighted by its interactions with both of these proteins. Glutathione S-transferase (GST) pulldown assays using parkin deletion constructs have mapped BAG5 interactions with parkin to both parkin's N-terminal linker region, and its C-terminal IBR and RING2 domains (Kalia et al., 2004). BAG5 negatively regulates parkin, inhibiting its E3 ligase activity and autoubiquitination (Kalia et al., 2004). Reciprocal GST pulldowns with BAG5 and PINK1 deletion constructs indicate that the

three N-terminal and the C-terminal BAG domains, but not the fourth BAG domain can interact with PINK1 (Wang et al., 2014). Interestingly, full-length GST-BAG5 pulls down both full-length and PARL-processed PINK1 but any lone BAG domain can only interact with full-length PINK1 (Wang et al., 2014). PINK1 interacts with BAG5 through its kinase domain (Wang et al., 2014).

BAG5 also plays a role in regulating toxic α-synuclein species. Like other BAG family members, BAG5 acts as a nucleotide exchange factor for Hsp70, promoting the release of ADP from the NBD and inhibiting Hsp70 refolding capacity, a process known to protect against α-synuclein toxicity (Auluck et al., 2002; Höhfeld and Jentsch, 1997; Kalia et al., 2004). BAG5 indirectly impairs the clearance of the α-synuclein oligomers by preventing its ubiquitination by another E3 ubiquitin ligase, C-terminal Hsp70-interacting protein (CHIP) (Kalia et al., 2011). By inhibiting E3 ubiquitin ligases, BAG5 may also play a role in another neurodegenerative disease that can present with a parkinsonian phenotype, spinocerebellar ataxia type-3, by preventing the ubiquitination pathogenic mutants of ataxin3 that contain expanded polyglutamine domains, , (Che et al., 2015). The generally negative regulatory functions that BAG5 exerts on multiple parkinsonian gene products highlights its potential role in the etiology of PD.

FIGURE 2

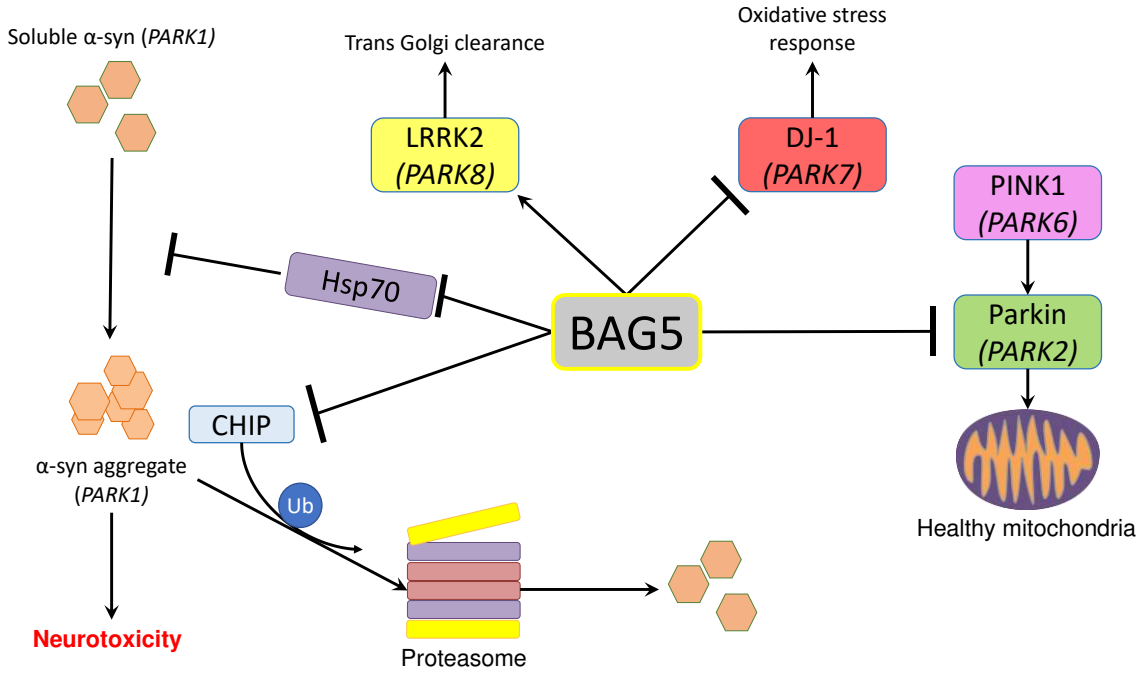

Figure 2: Schematic showing interactions between BAG5 and parkinsonian gene products. BAG5 inhibits the refolding activity of Hsp70 (Kalia et al., 2004), which is known to reduce α-synuclein aggregation (Auluck et al., 2002). It also inhibits the E3 ligase, CHIP, which ubiquitinates α-synuclein oligomers for proteasomal degradation (Kalia et al., 2011). BAG5 is part of a large complex with LRRK2 which leads to enhanced clearance of the trans golgi complex (Beilina et al., 2014). BAG5 attenuates the protective capacity of DJ-1 in response to Complex I inhibition (Qin et al., 2017). BAG5 can interact with PINK1 (Wang et al., 2014) and impairs parkin's E3 ubiquitin ligase activity (Kalia et al., 2004).

0.7 Research Objectives

The purpose of this study is to understand if the interactions between BAG5 and the parkinsonian gene products, PINK1 and parkin, affect mitophagy. Given that other BAG proteins differentially regulate some aspects of the PINK1 parkin pathway (Hasson et al., 2013; Qu et al., 2015), and that BAG5 interacts with parkin and enhances neurodegeneration (Kalia et al., 2004), we hypothesize that BAG5 negatively regulates PINK1 dependent parkin translocation and mitophagy. The specific aims were as follows:

Objective 1: Determine if BAG5 affects accumulation of PINK1

Objective 2: Determine if BAG5 decreases the translocation of parkin to the mitochondria following mitochondrial depolarization

Objective 3: Determine functional consequences of BAG5 on mitophagy

Chapter 1 BAG5 Interacts with PINK1 and Affects its Accumulation

1.1 Introduction

As described in the introduction of this thesis, it has been previously shown that BAG5 can interact with PINK1 (Wang et al., 2014). In this chapter we confirm this interaction and show that it is dependent on having functional BAG domains, as the BAG5 mutant BAG5-DARA cannot bind PINK1. BAG5-DARA is a mutant where the interaction with Hsp70 is abolished (Kalia et al., 2004). This is achieved by mutating key aspartate residues in α-helix 2 and arginine residues in α-helix 3 in four of the total five BAG domains to alanine (Kalia et al., 2004). We also test the hypothesis that BAG5 affects PINK1 accumulation following mitochondrial depolarization.

1.2 Materials and Methods

1.2.1 Cell Culture

Human osteosarcoma U2OS cell stably expressing GFP or GFP-parkin were a gift from Dr. Edward Fon (Montreal Neurological Institute and Hospital, McGill University) (Durcan et al., 2014). U2OS cells were maintained at 37°C and 5% CO_2 in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), and 1% penicillin/streptomycin (Life Technologies).

1.2.2 Transfection

For plasmid transfections, cells were plated and incubated overnight. Transfection mixtures were prepared in Opti-MEM (Life Technologies) containing plasmid DNA, and Lipofectamine 2000 (Invitrogen) according to manufacturer's directions and added to cell cultures. Cells were treated/harvested 24 hours post-transfection.

1.2.3 Co-immunoprecipitation

U2OS GFP cells were transfected with combinations of vectors expressing C-terminal myc-tagged PINK1 (PINK1-myc), N-terminal Flag-tagged BAG5 (FlagBAG5), and N-terminal Flag-tagged mutant BAG5-DARA (FlagDARA) for 24 hours before lysis in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100) with protease inhibitor cocktail (Roche). Cell lysates were incubated for 4 hours at 4°C with agarose beads conjugated with anti-Flag M2 antibodies (Sigma). Beads were washed in RIPA buffer supplemented with 0.1% SDS 5 times before elution with Flag peptide in TBS for 30 min at 4°C in agitation. Samples were then separated by SDS-PAGE and analysed by Western blot.

1.2.4 CCCP Treatment

U2OS cells were treated with 20 μM carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (Sigma) in DMEM to abolish mitochondrial membrane potential ($\Delta\psi m$) for the indicated lengths of time.

1.2.5 Western Blotting

Cells were harvested and lysed by radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Roche). Whole cell lysates or immunoprecipitations were prepared with SDS-PAGE sample buffer, boiled for 10 minutes, and run on SDS-PAGE gels, and transferred on to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skim milk in 0.1% TBS-Tween (TBST) and probed with antibodies for PINK1 (Cell Signaling Technologies, 1:500 or 1:250), Hsp70 (Enzo, 1:500), Flag M2 (Sigma 1:2000), or actin (Sigma, 1:2000) for 1 hour at room temperature or overnight at 4°C and visualized with Amersham ECL substrate (GE Healthcare) or Clarity ECL substrate (BioRad) and exposure to autoradiographic film.

1.3 Results

1.3.1 BAG5, but not BAG5-DARA, can interact with PINK1

To confirm that BAG5 is able to interact with PINK1 in our system, immunoprecipitations using antibodies directed towards the Flag epitope were performed from U2OS cell lysates that were transfected with combinations of plasmids expressing PINK1-myc, FlagBAG5, and the BAG5 mutant, FlagDARA, which does not bind Hsp70. Immunoprecipitation of FlagBAG5, but not FlagDARA, resulted in the co-immunoprecipitation of PINK1-myc (Figure 3). Both 62 kDa and 52 kDa PINK1-myc protein was co-immunoprecipitated with FlagBAG5, indicating that BAG5 can interact in a complex with both full-length and cleaved PINK1 (Figure 3). FlagDARA immunoprecipitation did not co-immunoprecipitate PINK1-myc, indicating that the BAG5-PINK1 interaction may be mediated electrostatic interactions with α-helices 2 or 3 domains that are disrupted by the aspartate and/or arginine mutations, or requires a complex with Hsp70.

FIGURE 3

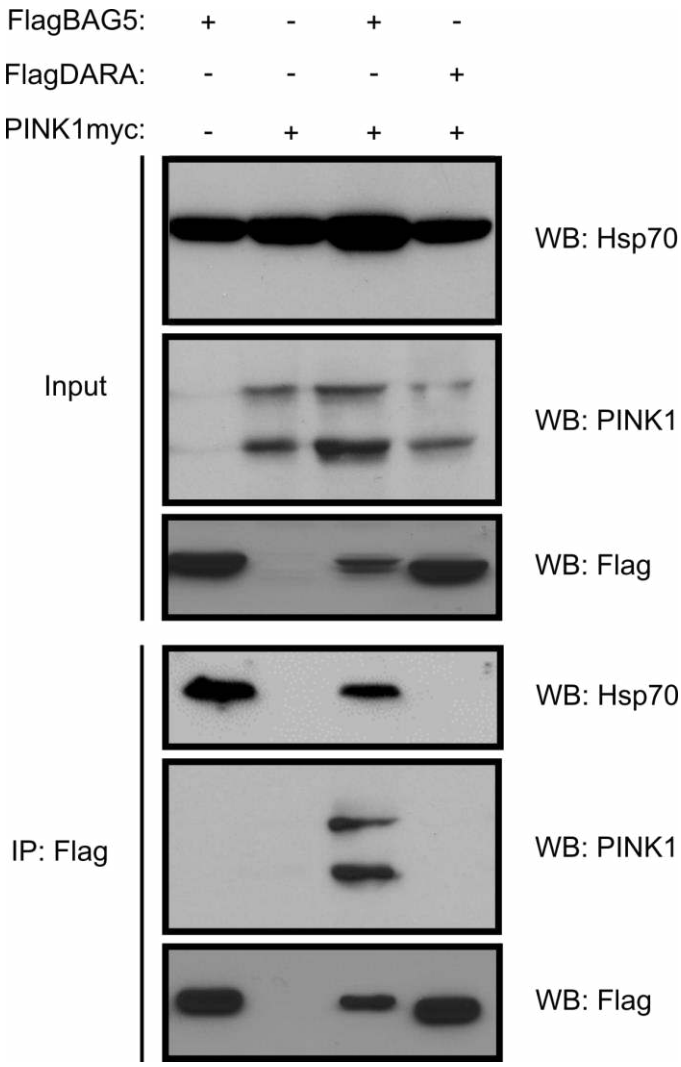

Figure 3: Immunoprecipitation of FlagBAG5, but not mutant FlagDARA, co-immunoprecipitates PINK1-myc. U2OS GFP cells were transfected with the indicated combinations of FlagBAG5, FlagDARA, and PINK1-myc. Cell lysates were incubated agarose beads conjugated with anti-Flag M2 antibodies and subsequently Western blotted for Flag, Hsp70 and PINK1. FlagBAG5 immunoprecipitation co-immunoprecipitated both full-length and processed PINK1.

1.3.2 BAG5 overexpression does not affect PINK1 accumulation following CCCP treatment

To evaluate consequences of the BAG5-PINK1 interaction, the levels of PINK1 protein were assessed following dissipation of mitochondrial membrane potential with the protonophore, CCCP, a known trigger of PINK1 accumulation (Narendra et al., 2010b). U2OS GFP-parkin cells (Durcan et al., 2014) were transfected with empty vector (pcDNA), or plasmids expressing FlagBAG5 or FlagDARA and treated with CCCP for the indicated durations. Western blotting revealed no difference in PINK1 accumulation between cells transfected with pcDNA, FlagBAG5, or FlagDARA (Figure 4).

FIGURE 4

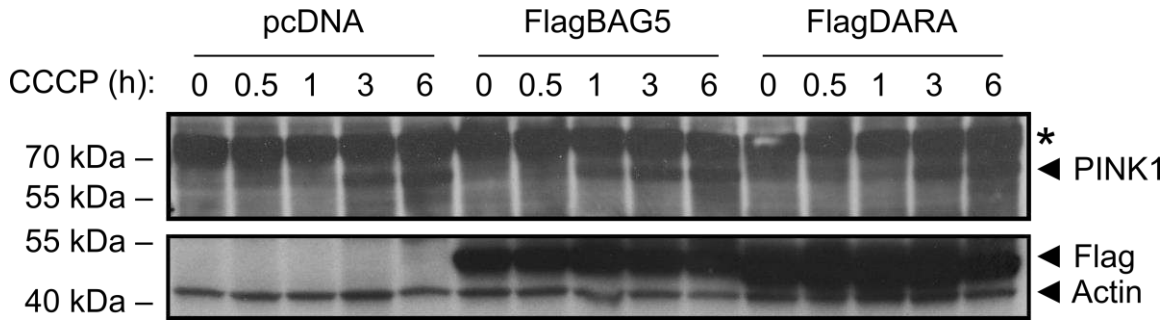

Figure 4: FlagBAG5 overexpression does not affect PINK1 accumulation following mitochondrial depolarization. FlagBAG5, nor FlagDARA, enhanced the accumulation of full-length PINK1 protein following 20 μM CCCP treatment. Full length PINK1 is 62 kDa, * indicates non-specific band. Western blot is representative of 3 independent experiments.

1.4 Discussion

Here we have confirmed the purported interaction between BAG5 and PINK1 (Wang et al., 2014), and shown that this interaction is dependent on having functional BAG domains as PINK1 co-immunoprecipitates with BAG5 but not with mutant BAG5-DARA (Figure 3). PINK1 has previously been shown to interact with Hsp70 (Rakovic et al., 2011; Zheng et al., 2018).

Given that the mutant, BAG5-DARA, cannot bind Hsp70 and does not co-immunoprecipitate PINK1, it is likely that BAG5's interaction with PINK1 is mediated by Hsp70.

The accumulation of PINK1 on mitochondria is an essential upstream step in sensing damaged mitochondria and triggering mitophagy (Pickrell and Youle, 2015). Here we have observed that FlagBAG5 overexpression does not affect PINK1 accumulation after mitochondrial depolarization (Figure 4). The other BAG family members known to affect the PINK1/parkin pathway, BAG2 and BAG4, have been examined for their effects on PINK1 in different contexts (Hasson et al., 2013; Qu et al., 2015). BAG4 was originally identified as a regulator of parkin recruitment to mitochondria and only afterwards was its effect on PINK1 assessed. It was determined that, like BAG5, it did not affect PINK1 accumulation following CCCP treatment, and it is believed to affect parkin by some other mechanism (Hasson et al., 2013). BAG2, on the other hand, was found to enhance the stability of PINK1 under untreated conditions by decreasing its ubiquitination (Qu et al., 2015). Qu et al. (2015), however, did not examine accumulation of full-length PINK1 in response to mitochondrial depolarization so it is hard to know if slowing the degradation of PARL-processed, 52 kDa PINK1 fragments influenced full-length mitochondrially stabilized PINK1.

Despite not impacting PINK1 accumulation, BAG4, was shown to interact with parkin and impairs its recruitment to mitochondria (Hasson et al., 2013). In contrast, BAG2 is believed to accelerate parkin's recruitment to mitochondria (Qu et al., 2015), but its ability to interact with parkin is unknown. Given that BAG5 is also known to interact directly with parkin, and inhibits parkin's autoubiquitination function (Kalia et al., 2004), we hypothesize that despite not affecting PINK1 accumulation following mitochondrial depolarization, BAG5 negatively regulates parkin recruitment to depolarized mitochondria.

Chapter 2 BAG5 Impairs Parkin Recruitment

2.1 Introduction

In the previous chapter, we demonstrated that BAG5 interacts with PINK1 in cells and did not affects its accumulation following CCCP treatment. Considering this effect and the fact that the other BAG family members, BAG2 (Qu et al., 2015) and BAG4 (Hasson et al., 2013), differentially impact parkin recruitment we investigated the hypothesis that BAG5 regulates parkin recruitment to mitochondria. Multiple imaging techniques were used to evaluate the effect of BAG5 knockdown and overexpression on GFP-parkin recruitment to CCCP depolarized mitochondria.

2.2 Materials and Methods

2.2.1 RNA Interference

RNAi was used to reduce BAG5 and BAG4 protein levels. Short interfering RNA targeting BAG5 (siBAG5)(Ambion, #s18285), BAG4 (siBAG4)(Ambion, #s18289), or non-targeting control (siNTC)(Ambion) were transfected into U2OS GFP-parkin cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to manufacturers instructions for reverse transfection.

2.2.2 Live Cell Time-Lapse Microscopy

U2OS GFP-parkin cells were plated into 8-well Nunc Lab-Tek II chambered coverglass (Thermo Fisher Scientific) containing siRNA (final concentration 10 nM), Lipofectamine RNAiMAX, and OptiMEM 48 hours before imaging. Media was changed 24 h after plating. Cells were infected with CellLight mitochondria-RFP (Thermo Fisher Scientific) 16 h prior to imaging to visualize the mitochondria. Microscopy was performed on a Nikon A1R scanning confocal microscope

equipped with an incubator maintained at 37°C, and 5% CO_2. Images were acquired using a 20x 0.75 NA CFI Plan Apo VC lens with GFP and RFP excited by 488 nm and 561 nm laser lines, respectively. Fluorescence images were acquired at 5-min intervals for 120 min.

2.2.3 Fixed Cell Immunocytochemistry

U2OS GFP-parkin cells were grown overnight in Nunc Lab-Tek II 8-well chamber slides (Thermo Fisher Scientific) before transfection with dsRed, FlagBAG5, or FlagDARA. At 24 hours post-transfection, cells were treated with 20 μM CCCP (Sigma Aldrich) for the indicated lengths of time. Cells were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.2% Triton X-100 in PBS, and blocked in 5% BSA in PBS at room temperature. Cells were incubated overnight at 4°C with primary antibodies for Flag (M2, Sigma) and TOMM20 (Abcam) and then with Alexa Flour (Thermo Fischer Scientific) conjugated secondary antibodies for 1 hour at room temperature. DAPI was incorporated into the final PBS wash at a final concentration of 300 nM. Cells were coverslipped with anti-fade fluorescent mounting medium (Dako). Images were acquired using an LSM 880 laser scanning confocal microscope (Zeiss) using a 20x, 0.75 NA, or 63x, 1.42 NA, lens (Advanced Optical Microscopy Facility at Wright Cell Imaging Facility). GFP-Parkin recruitment to mitochondria was quantified by the visualization of punctate GFP-Parkin that colocalized with the mitochondria marker, TOMM20.

2.2.4 Statistical Analysis

For analysis of live cell time-lapse imaging experiments, a minimum of 200 cells per condition were scored by a blinded counter for co-localization between GFP-parkin and mito-RFP across three independent experiments, each performed in triplicate (siNTC and siBAG5 conditions), or duplicate (siBAG4 conditions). For statistical analysis, a two-way ANOVA followed by

Bonferroni post-test was applied to determine significance. ***p<0.001 compared to siNTC, †p<0.001 compared to siBAG5.

For analysis of fixed cell overexpression experiments, a minimum of 150 transfected cells were scored for co-localization between GFP-parkin and the mitochondrial marker, TOMM20, across three independent experiments. For statistical analysis, one-way ANOVA followed by Tukey post-hoc test was performed. *p<0.05, ns not significant

2.3 Results

2.3.1 BAG5 siRNA Mediated Knockdown Accelerates Parkin Recruitment to Depolarized Mitochondria

U2OS GFP-parkin were transfected with non-targeting control, or siRNA targeting BAG5 or BAG4. BAG4 was included as a positive control for enhanced parkin recruitment (Hasson et al., 2013). Efficient knockdown of BAG5 and BAG4 protein levels was confirmed by Western blot (Figure 5). siRNA transfected cells were imaged live at 5-minute intervals for 2 h after addition of CCCP to a final concentration of 20 μM (Figure 6). Quantification of the percentage of cells displaying co-localization between GFP-parkin and mitochondria-RFP was performed by a blinded counter. Both BAG5 and BAG4 siRNA were found to significantly enhance the percentage of cells displaying GFP-parkin recruitment at timepoints from 30 to 60 minutes inclusive after CCCP treatment (Figure 7). BAG4 siRNA was found to accelerate GFP-parkin recruitment to an even greater degree than BAG5 siRNA, with a significantly higher proportion of siBAG4 transfected cells showing GFP-parkin recruitment at 30 and 40 minutes than siBAG5 transfected cells. The fact that BAG5 knockdown accelerates GFP-parkin recruitment indicates that BAG5's normal function is to inhibit parkin recruitment.

FIGURE 5

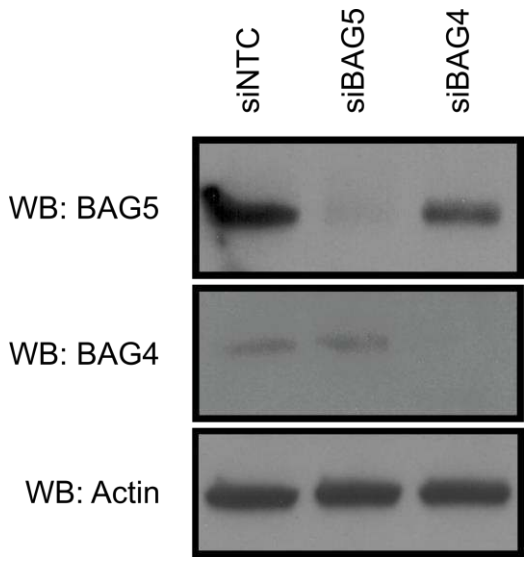

Figure 5: Western blot confirming efficient knockdown of BAG5 and BAG4 protein levels. U2OS GFP-parkin cells were transfected with non-targeting, BAG5, or BAG4 siRNA (10 nM) for 48 h. Cells were lysed and analyzed by Western blot for BAG5, BAG4, and actin.

FIGURE 6

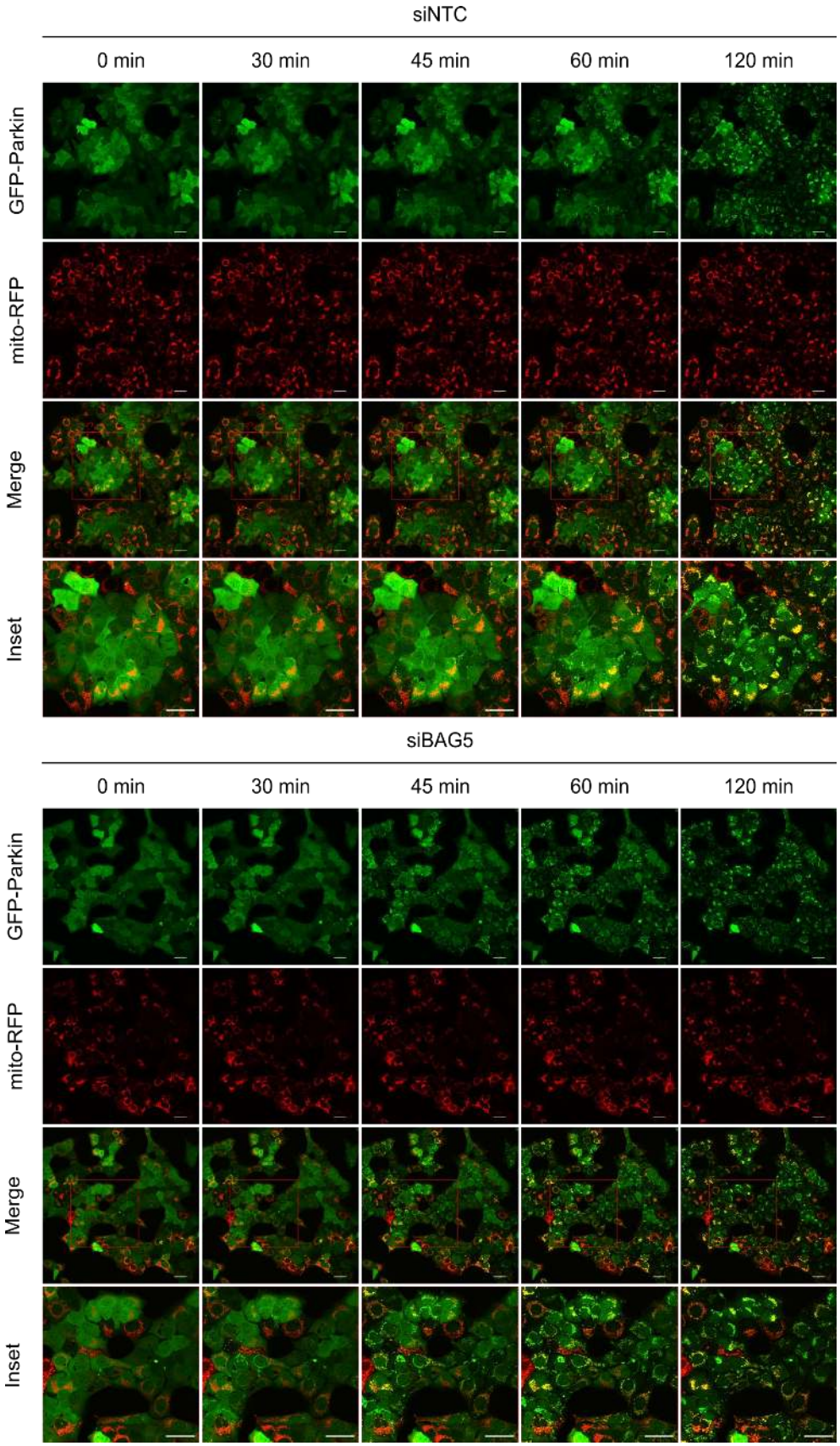
siNTC
0 min
30 min
45 min
60 min
120 min
GFP-Parkin
mito-RFP
Merge
Inset
siBAG5
0 min
30 min
45 min
60 min
120 min
GFP-Parkin
mito-RFP
Merge
Inset

FIGURE 6 CONT'D

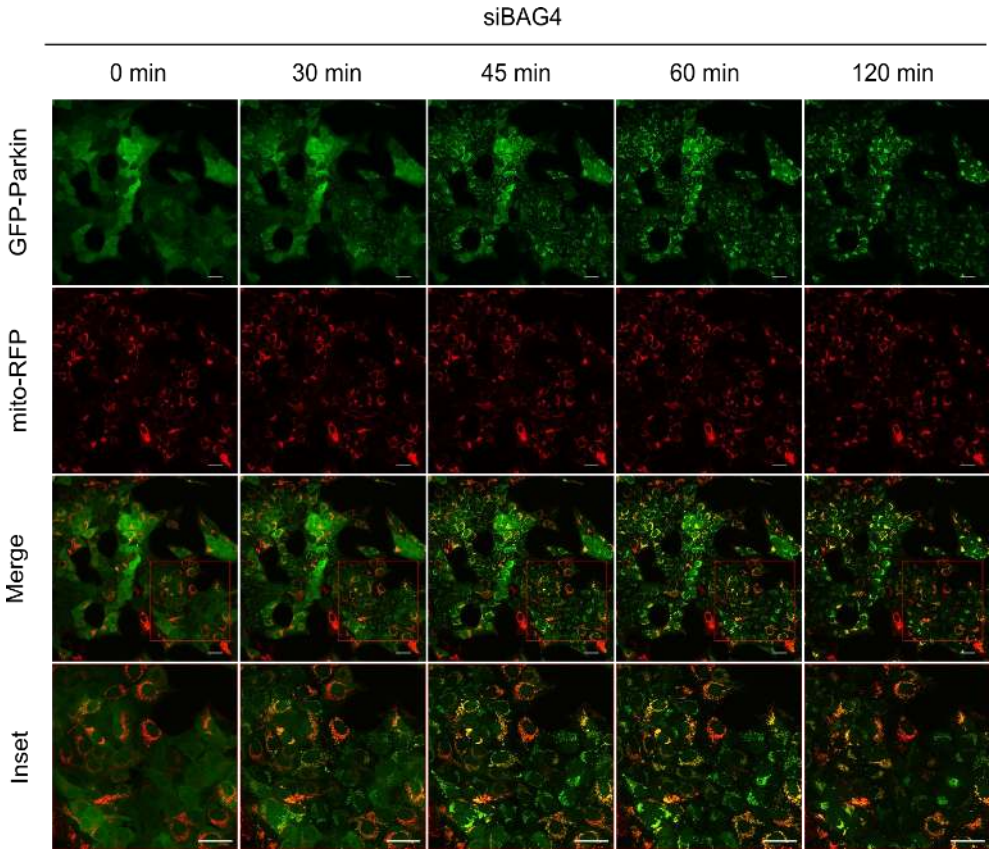

Figure 6: BAG5 siRNA-mediated knockdown accelerates parkin recruitment to mitochondria. Representative confocal micrographs of 2 h live cell time-lapse imaging of U2OS GFP-parkin cells treated with 20 μM CCCP and transfected with non-targeting, BAG5, or BAG4 siRNA 48 h prior to imaging and infected with CellLight mitochondria-RFP 16 h prior to imaging. Scale bar is 50 μm.

FIGURE 7

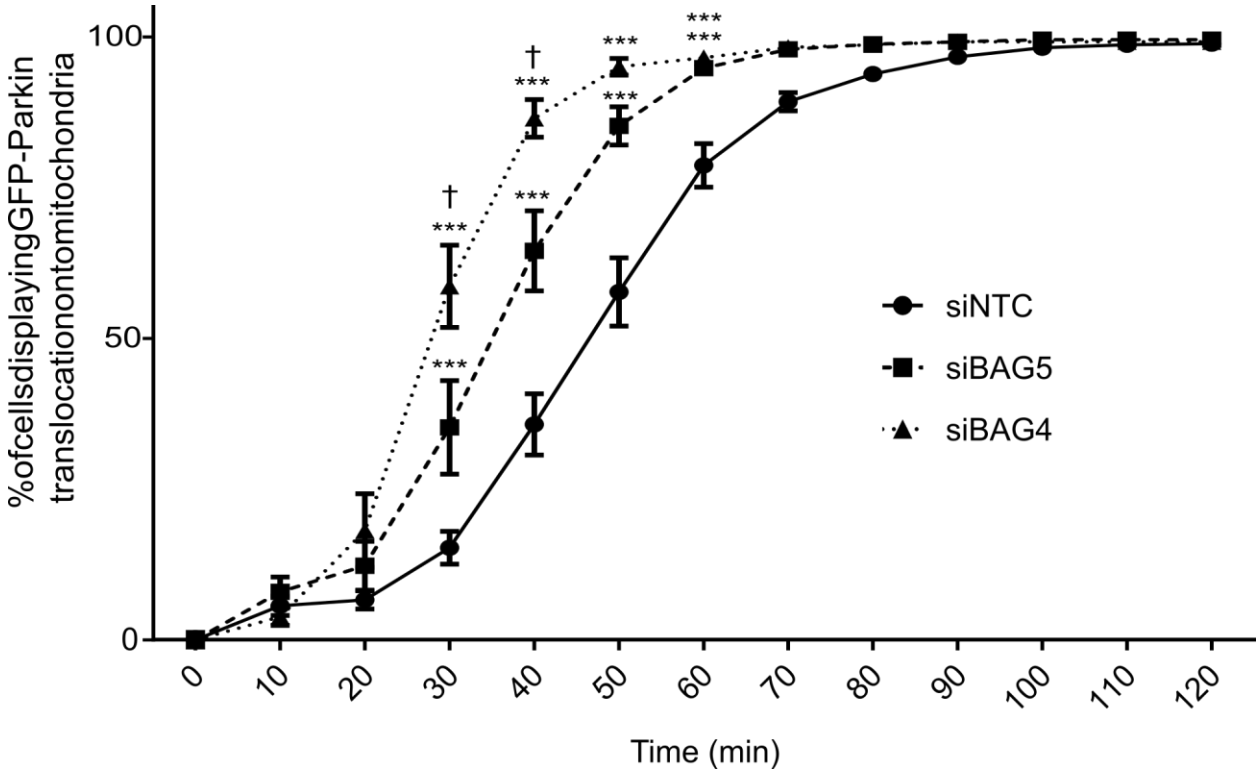

Figure 7: Quantification of GFP-parkin recruitment to mitochondria after siRNA-mediated knockdown. Quantification was performed by a blinded counter evaluating the percentage of cells displaying co-localization between GFP-parkin and mito-RFP. Experiments were performed in triplicate (siNTC and siBAG5 conditions), or duplicate (siBAG4 condition) with at least 200 cells counted per condition. The vertical bars represent the mean ± SEM for 3 independent experiments. Two-way ANOVA followed by Bonferroni post-test revealed siBAG5 and siBAG4 have significantly greater percentage of cells displaying GFP-parkin translocation than siNTC at timepoints between 30 and 60 minutes inclusive (***$p<0.001$ compared to siNTC). siBAG4 also had a significantly higher percentage of GFP-parkin translocation than siBAG5 at 30 and 40 minutes (†$p<0.001$).

2.3.2 FlagBAG5 Overexpression Delays, but does not Abolish, GFP-Parkin Recruitment to Depolarized Mitochondria

Next, we investigated the effect of BAG5 on GFP-parkin recruitment in an overexpression paradigm. Since BAG5 knockdown accelerated GFP-parkin recruitment to depolarized mitochondria, we hypothesized that BAG5 overexpression would have the opposite effect and impair GFP-parkin recruitment. Since siNTC transfected cells showed a large, but not complete proportion of cells with GFP-parkin recruitment to mitochondria at 60 minutes, we selected this timepoint for its dynamic range that could detect both increased or decreased parkin recruitment. To control for the selection of transfected cells when counting GFP-parkin recruitment, dsRed was used instead of empty vector for comparison with FlagBAG5 and FlagDARA transfection conditions.

U2OS GFP-parkin cells were transfected with dsRed, FlagBAG5, or FlagDARA and treated with 20 μM CCCP for 60 min before being fixed and stained for Flag and the mitochondrial marker, TOMM20 (Figure 8). Quantification of the percentage of transfected cells exhibiting co-localization between GFP-parkin and TOMM20 in each condition revealed that FlagBAG5 overexpression did indeed impair GFP-parkin recruitment at this timepoint (Figure 9). Interestingly, overexpression of FlagDARA, the BAG5 mutant incapable of binding Hsp70 and PINK1, but capable of binding wild-type BAG5 and parkin, also impaired GFP-parkin recruitment to the same degree as wild-type FlagBAG5 (Figure 9).

FIGURE 8

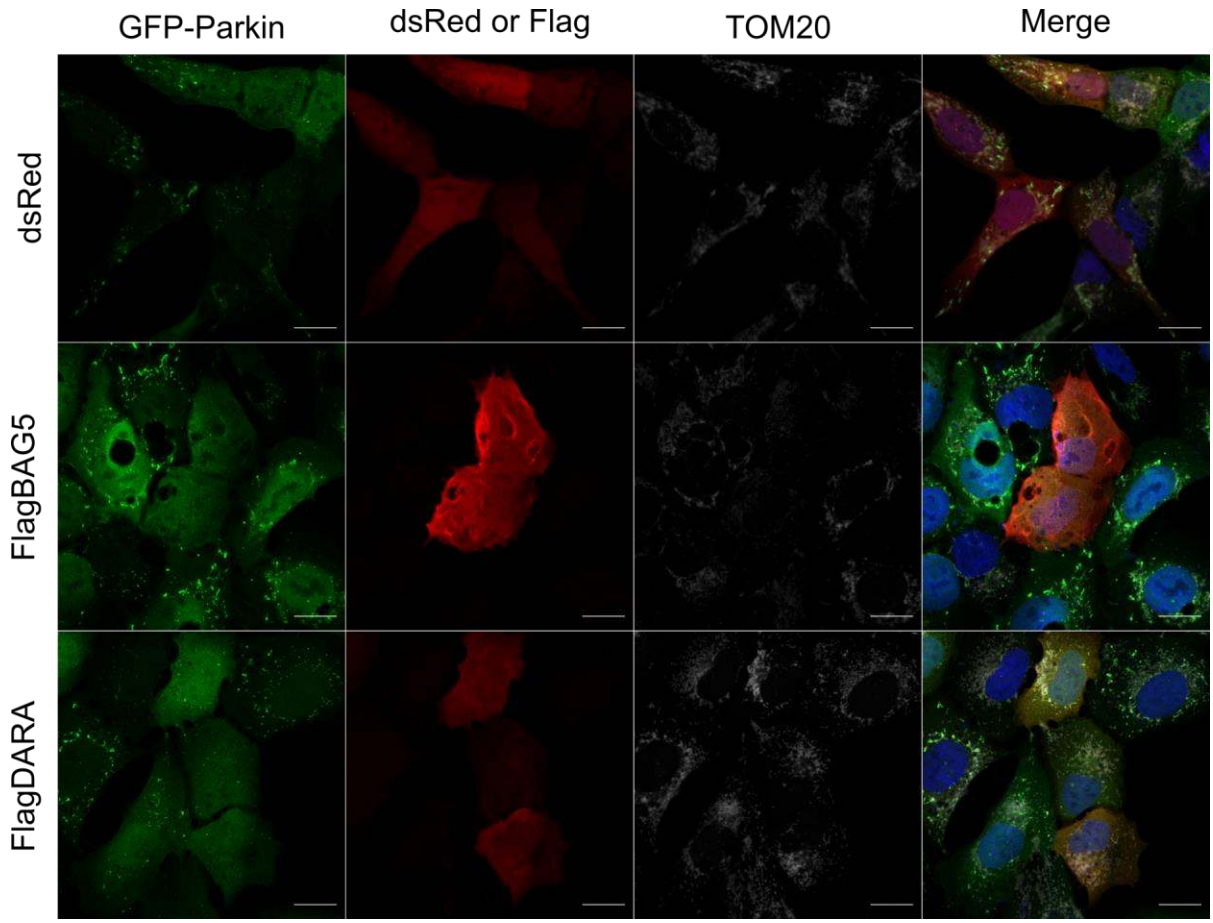

Figure 8: FlagBAG5 and FlagDARA impair GFP-parkin recruitment to mitochondria at 60 minutes after CCCP treatment. Representative confocal micrographs of U2OS GFP-parkin cells transfected with dsRed, FlagBAG5, or FlagDARA and treated with CCCP. Cells were transfected for 24 h before treatment with 20 μM CCCP for 60 minutes. They were then fixed, and immunostained for the mitochondrial marker, TOMM20, and imaged. Scale bar is 20 μm.

FIGURE 9

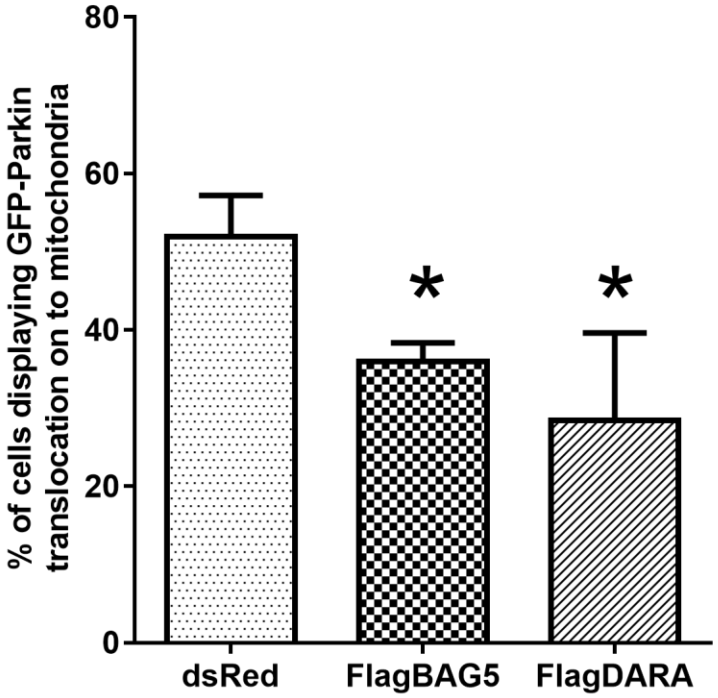

Figure 9: Quantification of GFP-parkin translocation after dsRed, FlagBAG5, or FlagDARA overexpression 60 min after CCCP treatment. Quantification was performed by evaluating the percentage of transfected cells displaying co-localization between GFP-parkin and the mitochondrial marker TOMM20. A minimum of 150 cells were counted per experiment. The vertical bars represent the mean ± SD for 3 independent experiments. For statistical analysis, one-way ANOVA followed by Tukey post-hoc test was performed, (*$p<0.05$).

To determine if the effect of FlagBAG5 overexpression is transient or persistent over the entire GFP-parkin recruitment time-course, the same fixed cell immunostaining paradigm was performed on U2OS GFP-parkin cells transfected with dsRed, FlagBAG5, or FlagDARA after 90 minutes of 20 μM CCCP treatment (Figure 10). Quantification of the percentage of transfected cells exhibiting co-localization between GFP-parkin and TOMM20 in each condition revealed that FlagBAG5, and FlagDARA overexpression no longer significantly impaired GFP-parkin recruitment at 90 min indicating that the initial effect is eventually overcome (Figure 11).

FIGURE 10

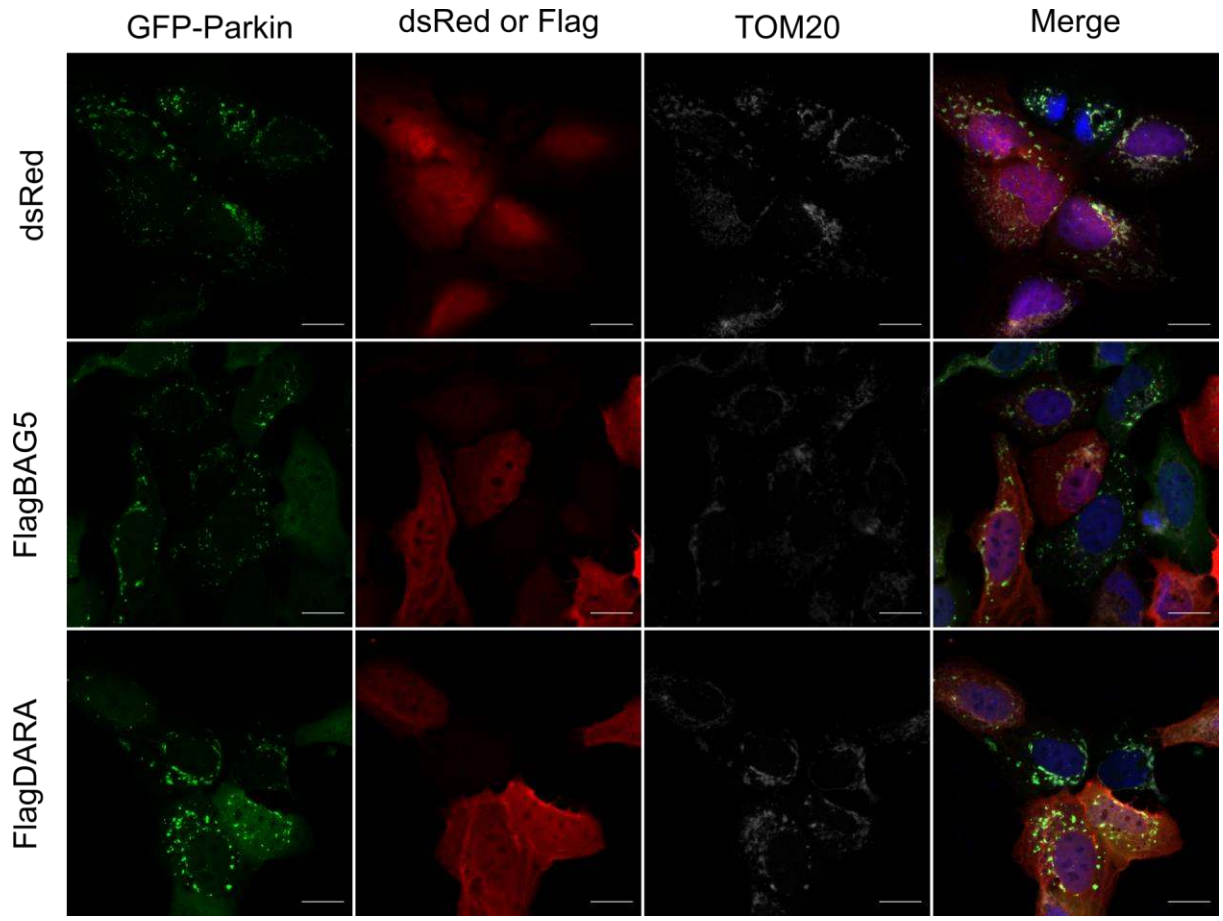

Figure 10: At 90 min after CCCP treatment GFP-parkin recruitment in FlagBAG5 and FlagDARA cells has caught up to dsRed control. Representative confocal micrographs of U2OS GFP-parkin cells transfected with dsRed, FlagBAG5, or FlagDARA and treated with CCCP. Cells were transfected for 24 h before treatment with 20 μM CCCP for 90 minutes. They were then fixed, and immunostained for the mitochondrial marker, TOMM20, and imaged. Scale bar is 20 μm.

FIGURE 11

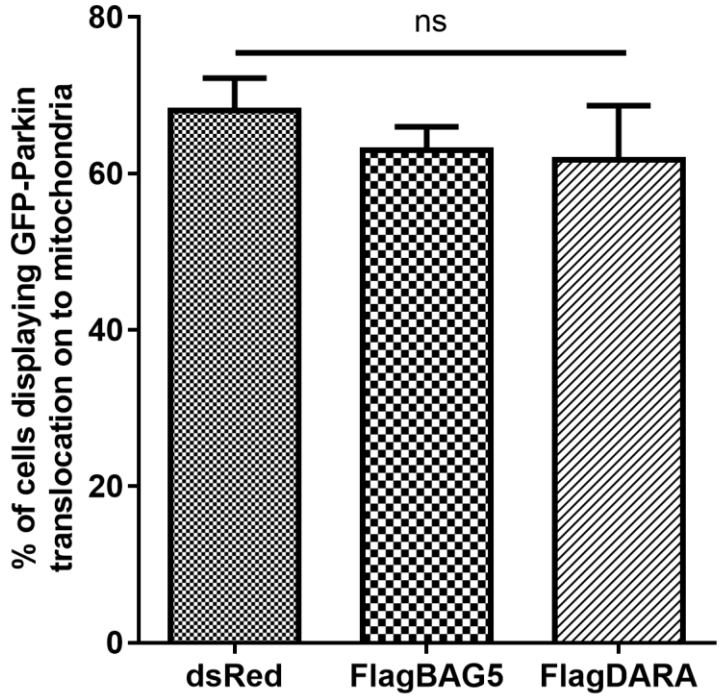

Figure 11: Quantification of GFP-parkin translocation after dsRed, FlagBAG5, or FlagDARA overexpression and 90 min CCCP treatment. Quantification was performed by evaluating the percentage of transfected cells displaying co-localization between GFP-parkin and the mitochondrial marker TOMM20. A minimum of 150 cells were counted per experiment. The vertical bars represent the mean ± SD for 3 independent experiments. For statistical analysis, one-way ANOVA followed by Tukey post-hoc test was performed. (ns, not significant)

2.4 Discussion

In this chapter we have shown that BAG5 is a novel negative regulator of parkin recruitment as its knockdown by RNAi accelerates GFP-parkin translocation to depolarized mitochondria and, conversely, the overexpression of FlagBAG5 delays GFP-parkin translocation (Figure 7-10). This places BAG5 in company with the two other BAG family members, BAG2 and BAG4, that have been shown to regulate parkin recruitment (Hasson et al., 2013; Qu et al., 2015). While

BAG2 purportedly accelerates parkin recruitment (Qu et al., 2015), BAG5 acts like BAG4, and impedes parkin recruitment (Hasson et al., 2013). The differences between the effects of BAG5 and BAG4 compared to BAG2 may be a result of different interactions between parkin and/or other proteins mediated by the shortened BAG domains of BAG5 and BAG4 (Briknarová et al., 2002) compared to BAG2's structurally unique C-terminal brand new BAG (BNB) domain (Xu et al., 2008). Indeed, both BAG4 and BAG5 interact with parkin (Hasson et al., 2013; Kalia et al., 2004). An interaction between BAG2 and parkin has not been demonstrated, but it is known to interact with the and inhibit another E3 ligase, CHIP (Arndt et al., 2005).

Interestingly, FlagDARA overexpression also impaired GFP-parkin translocation to the same degree as wild type FlagBAG5 after 60 minutes of CCCP treatment (Figure 9). This suggests that BAG5's ability to regulate parkin recruitment is independent of its interaction with PINK1, as FlagDARA is unable to interact with either full-length or processed PINK1 (Figure 3). While the mutations in BAG domains of BAG5-DARA disrupt Hsp70 binding and the PINK1 interaction, they do not, however, abolish BAG5's interaction with parkin (Kalia et al., 2004). Since BAG5 is known to interact with parkin's N-terminal linker region and C-terminal IBR and RING2 domains (Kalia et al., 2004), it is possible that the mechanism by which BAG5 impairs parkin recruitment is through stabilization of the one or both of the interactions between parkin's REP and RING1 or RING0 and RING2 domains, key hydrophobic interactions for maintaining parkin's autoinhibited state (Tang et al., 2017; Trempe et al., 2013). Even a slight stabilization of parkin's inhibited state could cause a strong attenuation of parkin recruitment by disrupting the feedforward nature of parkin recruitment where activated parkin provides more ubiquitin substrate for PINK1 to phosphorylate which in turn activates more parkin (Ordureau et al., 2014; Pickrell and Youle, 2015). Indeed, decreasing the ubiquitin chain density is an effective

mechanism that has been shown to impair parkin recruitment as the deubiquitinating enzymes, USP15 and USP30, delay parkin recruitment by cleaving polyubiquitin chains (Bingol et al., 2014; Cornelissen et al., 2014; Wang et al., 2015).

The overexpression GFP-parkin recruitment experiments in this chapter relied on dsRed as a transfection control. It was chosen as a non-specific protein that could both be used to identify transfected cells and as a control for the non-specific effects of overexpression. This is in contrast to other studies on modifiers of GFP-parkin that rely on non-expressing, empty vectors which control for the introduction of exogenous DNA by cationic lipid transfection but not for any off-target effects that may result from the overexpression of an inert protein (Hollville et al., 2014; Qu et al., 2015). While dsRed is not absolutely ideal because it does not contain the Flag tag that the BAG5 and DARA plasmid do, it is sufficient because Flag is only 8 residues long and engineered to be hydrophilic, so it is extremely unlikely to affect solubility or exhibit other off-target effects (Hopp et al., 1988). In an effort to address potential criticism over the use of dsRed as a control for comparison against Flag-tagged experimental conditions, these same experiments were attempted using BAG5 tagged with the blue fluorescent protein, TagBFP, with TagBFP alone as the control. However, these experiments were limited by insufficient expression of the TagBFP-BAG5 construct (Data not shown).

Taken together, the data presented in this chapter suggest that BAG5 is a negative regulator of parkin recruitment to depolarized mitochondria. Several other proteins that affect parkin recruitment including, ATPIF1 (Lefebvre et al., 2013), hexokinases 1 and 2 (McCoy et al., 2014), USP8 (Durcan et al., 2014), and USP30 (Bingol et al., 2014; Wang et al., 2015), have subsequently been shown to also affect mitophagy. In the next chapter, we test the hypothesis that impairment of parkin recruitment by BAG5 also leads to an impairment in mitophagy.

Chapter 3 BAG5 Impairs Mitophagy

3.1 Introduction

In the previous chapter, we showed that BAG5 knockdown by RNAi accelerated GFP-parkin recruitment to depolarized mitochondria, while FlagBAG5 and FlagDARA overexpression had the converse effect. In this chapter, we investigate the hypothesis that this effect on parkin recruitment will in turn impact mitophagy. We employed a sensitive and quantitative fluorescence automated cell sorting (FACS) assay that is based on the localization of a mitochondrially-targeted, monomeric variant of the fluorescent coral protein, Keima (mito-mKeima) (Katayama et al., 2011). Keima is a red fluorescent protein that exhibits a large pH-dependent change in its excitation spectrum as a result of the conformational change induced by the protonation of the aspartate residue at position 157 (Violot et al., 2009). Measuring the ratio between mito-mKeima emission in response to excitation by 405 nm wavelength (pH 7) and 561 nm wavelength (pH 4) laser light allows for differentiation between mito-mKeima that is localized to the neutral environment of the cytosol, and mito-mKeima that is engulfed in the acidic environment of the lysosome (Katayama et al., 2011). This allows for direct detection of mitochondria that are being degraded in lysosomes at any given time (Figure 12).

FIGURE 12

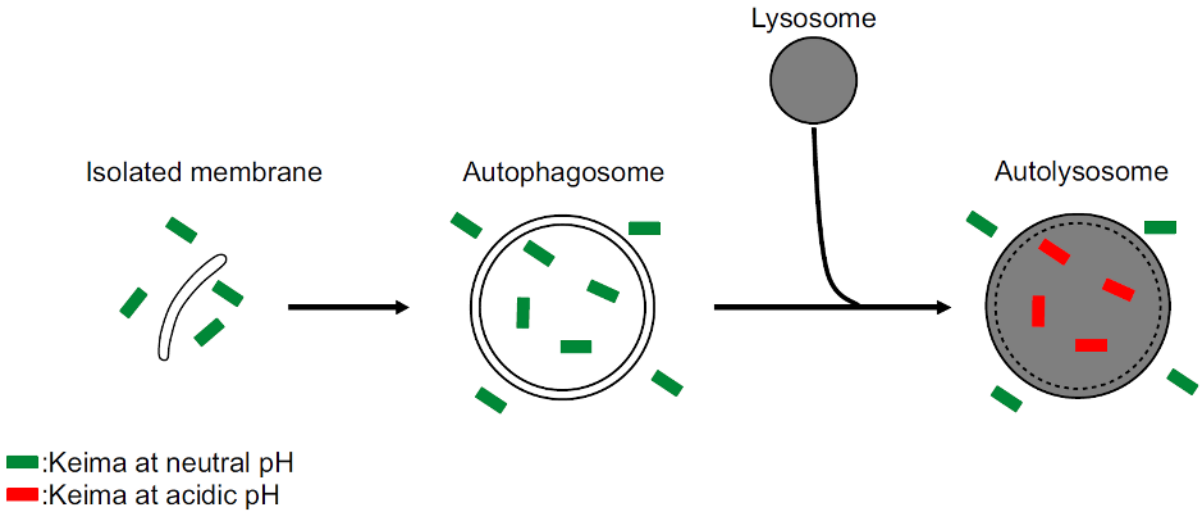

Figure 12: Schematic illustrating the change in mito-mKeima fluorescence in response to engulfment in the acidic environment of the lysosome. Reprinted from Chemistry & Biology, Vol 18, Issue 8, Katayama et al, A Sensitive and Quantitative Technique for Detecting Autophagic Events Based on Lysosomal Delivery, Pages 1042-1052, Copyright (2011), with permission from Elsevier.

3.2 Materials and Methods

3.2.1 Mitophagy Assay

U2OS cells stably expressing both GFP-parkin and ecdysone-inducible mito-mKeima were a gift from Dr. Edward Fon and Dr. Matthew Tang (Montreal Neurological Institute and Hospital, McGill University) (Tang et al., 2017). U2OS GFP-parkin/mito-mKeima cells were plated into 6-well plates containing siRNA (final concentration 10 nM), Lipofectamine RNAiMAX, and OptiMEM. Media was changed to DMEM containing 10 µM ponasterone A (Sigma) 24 h after plating to induce expression of mito-mKeima. Cells were treated with 20 µM CCCP for 4 hours at 48 h post-transfection. For flow cytometry analysis, cells were trypsinized, washed, resuspended in PBS and analyzed using a LSR Fortessa Cell Analyzer (BD Bioscience) equipped

with 405 and 561 nm lasers and 610/20 emission filter (Princess Margaret Hospital Flow Cytometry Facility). Lysosomal mito-mKeima was detected through a ratiometric pH measurement where pH 7 was detected by excitation with 405 nm laser and pH 4 was detected by excitation with 561 nm laser. A 610/20 emission filter was applied to detect emission resulting from both excitation wavelengths. For each sample, 50 000 events were collected, and cells were gated for GFP-parkin and mito-mKeima expression. Data were analyzed using FlowJo X using the approach previously described by Tang et al. (2017).

3.2.2 Statistical Analysis

For the mitophagy assay, a one-way ANOVA followed by Tukey's post-hoc test was performed on data from three independent experiments each performed in triplicate. ***$p<0.001$

3.3 Results

3.3.1 BAG5 siRNA Mediated Knockdown Enhances Mitophagy

U2OS GFP-parkin/mito-mKeima cells were transfected with non-targeting, BAG5, or PINK1 siRNA. PINK1 siRNA was included as a negative control for the mitophagy assay because it is necessary for parkin activation and mitophagy (Lazarou et al., 2015; Narendra et al., 2010b). Knockdown of PINK1 was confirmed by Western blotting (Figure 13). Treatment with 20 μM CCCP for 1 hour was necessary to induce sufficient levels of PINK1 accumulation to detect the difference between non-targeting control and PINK1 siRNA transfected conditions. siRNA transfected U2OS GFP-parkin/mito-mKeima cells were treated with 20 μM CCCP for 4 h before FACS analysis (Figure 14). Consistent with our result that BAG5 siRNA accelerates GFP-parkin recruitment, U2OS GFP-parkin/mito-mKeima cells transfected with BAG5 siRNA displayed a significantly greater proportion of cells with lysosomal positive mito-mKeima (Figure 15). This

indicates that at baseline, BAG5 acts to blunt mitophagy by impairing parkin recruitment to mitochondria. To confirm that the mitophagy observed in this assay is dependent on GFP-parkin activity, U2OS mito-mKeima cells also stably expressing catalytically inactive mutant GFP-parkin^{C431S} were also assessed (Figure 14). No increase in mitophagy was seen in these cells following CCCP treatment (Figure 15).

FIGURE 13

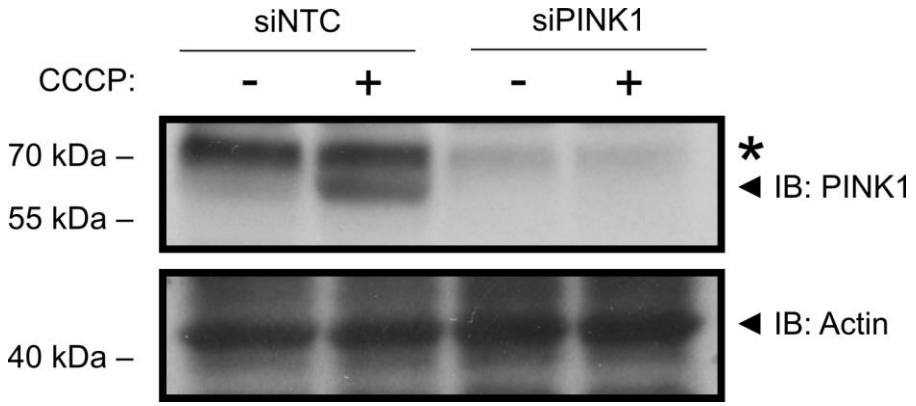

Figure 13: Western blot confirming efficient knockdown of PINK1 protein levels. U2OS GFP-parkin cells were transfected with non-targeting, or PINK1 siRNA for 48h and then treated with 20 μM CCCP or vehicle for 1 hr to induce sufficient levels of PINK1 to detect a difference between non-targeting and PINK1 knockdown conditions. Cells were lysed and analyzed by Western blot for PINK1 and actin. * indicates non-specific band.

FIGURE 14

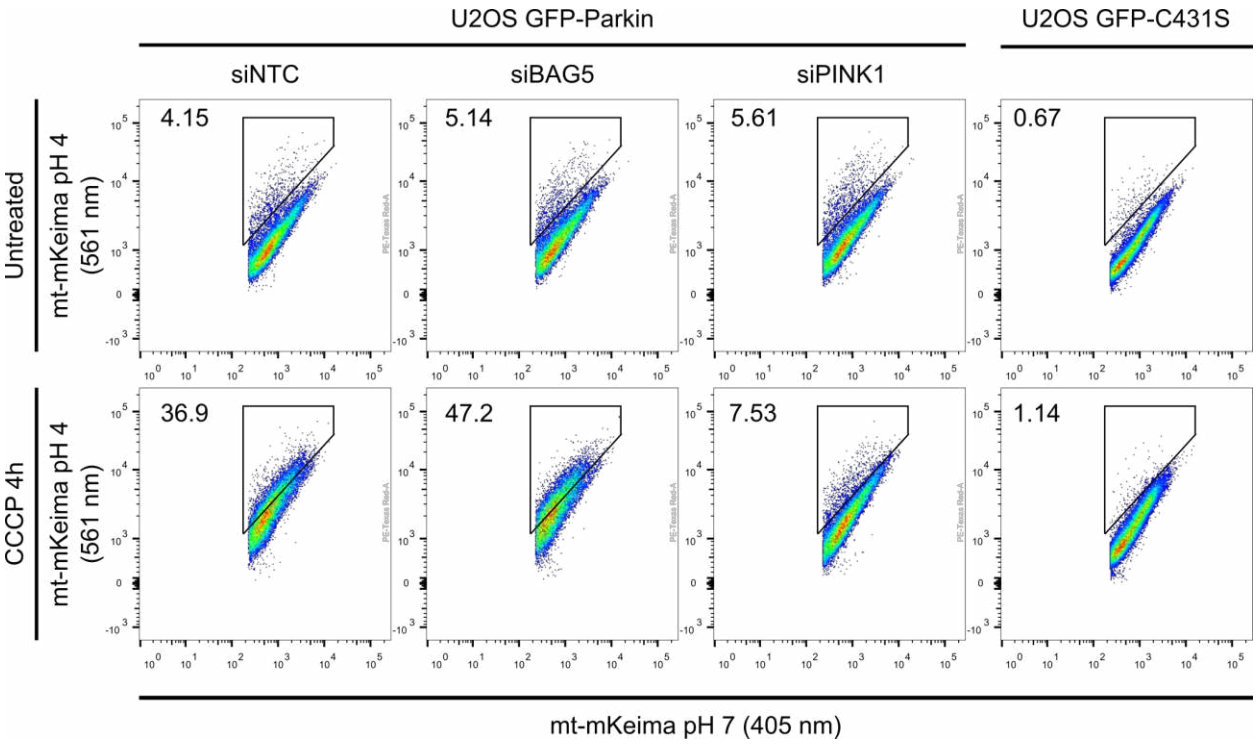

Figure 14: BAG5 siRNA-mediated knockdown enhances mitophagy. Representative FACS dot plots of U2OS mito-mKeima/GFP-parkin cells that were untreated or treated for 4 h with 20 μM CCCP 48 h after transfection with non-targeting, BAG5, or PINK1 siRNA. Lysosomal positive mito-mKeima are found in the upper gate and quantification is based on the percentage of cells in this lysosomal positive upper gate.

FIGURE 15

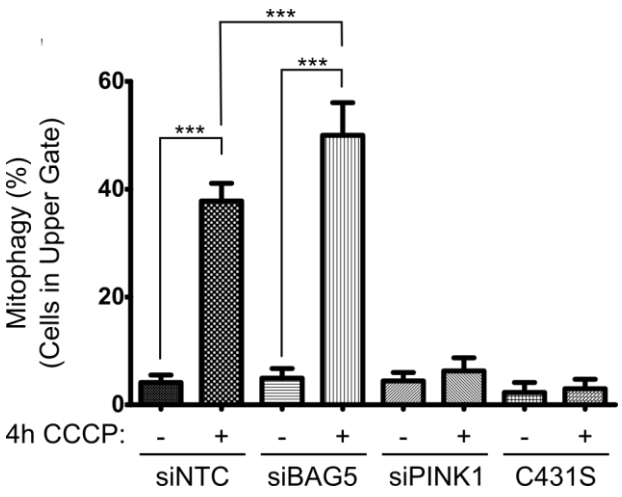

Figure 15: Quantification of average of mitophagy. The vertical bars represent the mean ± SD from three independent experiments each performed in triplicate. For statistical analysis, a one-way ANOVA with Tukey's post-hoc test was performed. *** $p<0.001$

3.4 Discussion

In this chapter we have shown that BAG5, consistent with its impairment of parkin recruitment, also impairs mitophagy. This indicates that BAG5 acts similarly to USP30, which impairs mitophagy by delaying parkin recruitment to mitochondria (Bingol et al., 2014; Wang et al., 2015). This is distinct from the DUBs, USP15 and USP35, which inhibit mitophagy but do not affect parkin recruitment (Cornelissen et al., 2014; Wang et al., 2015). The persistence of damaged mitochondria caused by BAG5's impairment of mitophagy may provides some insight into the mechanism through which BAG5 enhances neurodegeneration under certain circumstances (Kalia et al., 2004).

While mitochondria are essential for their role in cellular energy production and calcium buffering, they can have a detrimental impact on cellular viability through the production of ROS, initiation of the intrinsic apoptosis pathway, and initiation of necrosis (Green et al., 2011).

A number of steps in oxidative phosphorylation produce ROS, in the form of the superoxide anion (O_2^-) (Murphy, 2009). These ROS can initiate a vicious cycle where they damage mtDNA, further compromising oxidative phosphorylation leading to increased ROS production (Green et al., 2011). Furthermore, mitochondria harbour intracellular proteins such as cytochrome c, that when released into the cytosol, initiate the caspase dependent intrinsic apoptosis pathway (Tait and Green, 2010), or necrosis (Kinnally et al., 2011). Consistent with their role in maintaining healthy pools of mitochondria PINK1 and parkin protect against decreases in mitochondrial membrane potential, increased ROS production, impairments in respiration, and ATP synthesis (Gandhi et al., 2009; Gautier et al., 2012; Gautier et al., 2008; Heeman et al., 2011). By impairing the clearance of dysfunctional mitochondria by PINK1 and parkin dependent mitophagy, BAG5 increases the risk that they pose to the cell. This may explain the increased neurodegeneration that BAG5 causes following MFB axotomy and MPTP treatment (Kalia et al., 2004).

Mechanical lesion of the MFB, the projections that connect the SN to the striatum, causes acute and severe degeneration of the SN (Brecknell et al., 1995). While this model does not directly damage mitochondria, it does enhance ROS production in the SN (Venero et al., 1997), and the response to axonal injury involves enhanced mitochondrial flux to the site of injury and increased ATP production to support regeneration (Cartoni et al., 2016; Han et al., 2016). The persistence of deficient mitochondria lowers the neuron's ability to mount a successful response to injury, and increases the probability of cell death.

Neurodegeneration caused by MPTP is the direct result of inhibition of Complex I of the mitochondrial respiratory chain. PINK1 and parkin are both shown to be able to be protective against the dopaminergic neurodegeneration caused by this mitochondrial dysfunction (Bian et

al., 2012; Haque et al., 2008; Yasuda et al., 2011). Furthermore, their function in mitophagy is implicated in this protection against MPTP as upregulation of autophagy-related gene 5 (Atg5), a protein necessary for autophagosome formation, is also protective against MPTP toxicity (Hu et al., 2017). Therefore, impairment of PINK1 and parkin mediated mitophagy may be the mechanism through which BAG5 enhances neurodegeneration following MPTP treatment *in vivo*.

BAG5 may also enhance cell death by more direct means. Emerging evidence suggests that PINK1 and parkin may act as a molecular switch that can promote apoptosis in response to severe mitochondrial insult by promoting the proteasomal degradation of Mcl-1 (Carroll et al., 2014; Zhang et al., 2014). This hypothesis is reminiscent of other molecular switches like the oncogene, p53, which can promote DNA repair or apoptosis based on the severity of DNA damage (Kastan et al., 1991; Lowe et al., 1993). Mcl-1 is a Bcl-2 family member that normally prevents apoptosis by blocking the dimerization of components of the mitochondrial membrane transition pore that leads to cytochrome c release and caspase activation (Adams and Cory, 1998). It is possible that by impairing parkin recruitment to mitochondria, BAG5 allows parkin to ubiquitinate cytosolic substrates like Mcl-1. However, better understanding of how BAG5 prevents parkin recruitment to the mitochondria, and whether the cytosolic parkin is in a confirmation capable of E3 activity is necessary to speculate on this possibility.

Chapter 4 General Discussion and Future Directions

The aim of this thesis was to evaluate the role of BAG5 in PINK1 and parkin dependent mitophagy. Here we have confirmed that BAG5 is able to interact with both full length and processed PINK1, and show that this interaction is dependent on the function of the BAG domains. We also report that BAG5 is a novel regulator of parkin that hinders parkin's translocation from the cytosol to depolarized mitochondria. This delay in parkin recruitment by BAG5 was in turn shown to also impair mitophagy (Figure 16).

FIGURE 16

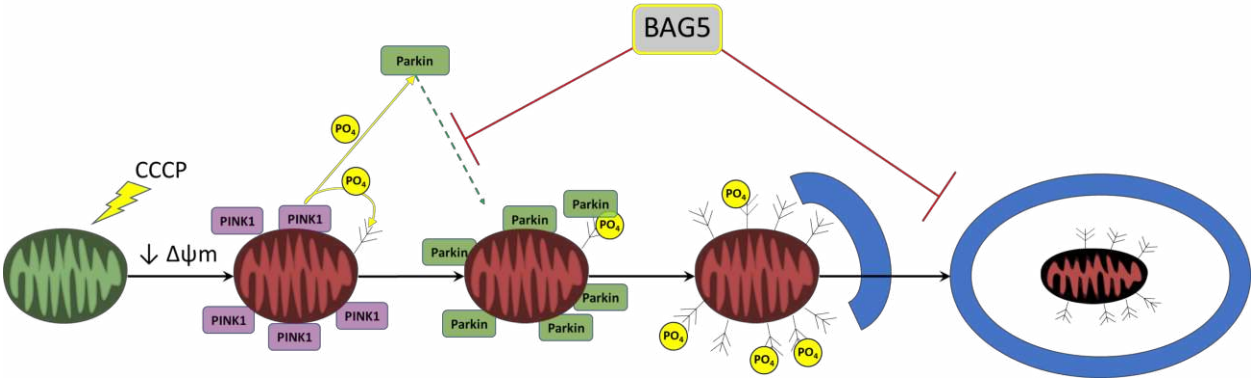

Figure 16: Schematic depicting the role of BAG5 in regulating PINK1 and parkin dependent mitophagy. BAG5 inhibits parkin recruitment to the mitochondria and impairs mitophagy.

Mitochondria are essential organelles that produce the majority of cellular ATP and are signalling hubs that integrate various cell death signals. Mitochondrial dysfunction has long been recognized as a component of several neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Lin and Beal, 2006). Evidence from mitochondrial toxins and genetics indicate that mitochondrial dysfunction directly causes parkinsonism (Bonifati et al., 2003; Kitada et al., 1998; Langston et al., 1983;

Valente et al., 2004). Considering that BAG5 enhances neurodegeneration in rodent models of PD (Kalia et al., 2004), interacts with several of other parkinsonian gene products (Figure 2), and impairs PINK1 and parkin dependent mitophagy, there is considerable evidence to suspect that BAG5 may play a role in the pathobiology of PD.

By impairing mitochondrial quality control, BAG5 may also contribute to the normal aging process. Age is the single greatest risk factor for PD and other neurodegenerative diseases, and mitochondria are hypothesized to play a causative role in the normal aging process (Bratic and Larsson, 2013). Mitochondrial number and function have long been known to decrease with age in humans (Tauchi and Sato, 1968; Yen et al., 1989), and the premature aging phenotype seen in mutator mice that accumulate mtDNA mutations due to defective mtDNA polymerase provides direct evidence for their causal role in aging (Kujoth et al., 2005; Trifunovic et al., 2004). Furthermore, the extension of life span by caloric restriction is believed to be at least partly due to enhanced mitochondrial function (Bratic and Larsson, 2013). The persistence of damaged mitochondria by inhibiting PINK1 and parkin directed mitophagy by BAG5 may play a role in promoting the normal aging process.

Interestingly, two other BAG family members, BAG1 and BAG3, are known to constitute an age and stress dependent expression switch that modifies protein quality control. BAG1 normally functions in protein quality control by promoting protein turnover through proteasomal degradation, however, with age or cellular stress its expression decreases and BAG3 expression is increased (Behl, 2016). BAG3 then causes a shift from protein turnover through proteasomal degradation to autophagic degradation (Behl, 2016). In the future it will be interesting to investigate if expression patterns of BAG5 and BAG2 change with age, potentially causing a similar functional switch in mitochondrial quality control by differentially regulating parkin.

Some of the limitations of this thesis include that only wholesale mitophagy was examined, all experiments were carried out in immortalized cell lines, and the mechanism by which BAG5 impairs parkin recruitment is not fully understood. These limitations leave open several avenues for future study including examining the role of BAG5 in MDV generation, mitophagy *in vivo,* and stabilization of parkin's inhibited state.

While wholesale mitophagy of mitochondrial networks is an important quality control mechanism, it may represent a response to severe mitochondrial damage and MDVs may be a more physiological response to moderate stresses (McLelland et al., 2016; McLelland et al., 2014). The complex III inhibitor, antimycin A, has been shown to model mitochondrial dysfunction and ROS production which is less severe than depolarization with ionophores and results in MDV formation (McLelland et al., 2014; Soubannier et al., 2012). Directly observing the formation of MDVs following antimycin A treatment using high-resolution confocal and/or electron microscopy in BAG5 overexpression and knockdown paradigms would allow for determination of its role in MDV formation. Considering that BAG5 impairs wholesale PINK1/parkin dependent mitophagy, we hypothesize that BAG5 would also impair MDV formation.

In future studies, it will be interesting to see if BAG5 also impairs PINK1/parkin dependent mitophagy *in vivo*. The recent development of two techniques for examining mitophagy *in vivo* will make this type of investigation possible (McWilliams et al., 2016; Sun et al., 2015). Both approaches rely on the use of fluorescent reporter proteins. The first system employs mito-mKeima transgenic mice (Sun et al., 2015). The second approach uses transgenic mice that express a tandem fluorescent construct, termed mitoQC, with mCherry and GFP fused to the MTS from the OMM protein, FIS1 (McWilliams et al., 2016). Like keima, mitoQC relies on pH

change to detect mitophagy. At cytosolic pH, both GFP and mCherry fluoresce, however, acidic pH quenches GFP fluorescence differentiating cytosolic and lysosomal localized mitoQC (Allen et al., 2013; McWilliams et al., 2016). While each system has advantages and disadvantages, either could be applied to study to the effect of BAG5 on mitophagy *in vivo.* Combining conditional knockouts of BAG5 or viral vector mediated modulation of BAG5 protein levels in specific areas of the mouse brain will make it possible to evaluates BAG5's effects *in vivo.*

The mechanism by which BAG5 impairs parkin recruitment has not been fully elucidated. Given its interactions with PINK1 and parkin there are a number of possible ways that BAG5 may affect the PINK1 and parkin dependent mitophagy pathway. Here we show that BAG5 overexpression does not affect PINK1 protein levels following mitochondrial depolarization by CCCP, indicating that altered PINK1 accumulation is not the mechanism for modulating parkin recruitment. Since BAG5 impairs mitophagy it is possible that the persistence of mitochondria in the cytosol at longer timepoints after depolarization actually could lead to higher full-length PINK1 levels as measured as a proportion of whole cell lysate.

Another possible function of the BAG5-PINK1 interaction that could impair parkin recruitment and mitophagy would be suppression of PINK1 phosphorylation of ubiquitin. With the recent development of specific phopsho-S65 ubiquitin antibodies (Fiesel et al., 2015), this possibility could be tested by quantifying the density of phospo-S65 ubiquitin on mitochondria following insult both in the presence and absence of BAG5. However, given that overexpression of FlagDARA, which cannot interact with PINK1, also impairs parkin to the same degree as wild-type FlagBAG5 another mechanism is more likely.

A possible mechanism by which BAG5 may delay parkin recruitment is through attenuating the strength of the feedforward cycle that leads to recruitment by hindering parkin's ubiquitin ligase activity. This could be achieved by stabilizing parkin's inhibited state. Consistent with this idea, BAG5 does inhibit parkin's E3 ligase activity as measured by its autoubiquitination activity (Kalia et al., 2004), as well as CHIP's E3 ligase activity (Kalia et al., 2011). BAG5 has been shown to interact directly with a number of parkin domains including the N-terminal linker, the IBR domain, and the C-terminal RING2 domain (Kalia et al., 2004). Kalia et al. (2004) performed this deletion construct interaction study before it was known that parkin contains a unique third zinc finger, RING0, to the N-terminal side of RING1 (Hristova et al., 2009), so the observed linker region interaction could actually represent an interaction with RING0. Furthermore, the REP domain is contained in the IBR domain deletion construct. These interactions suggest the possibility that BAG5 can stabilize one or both of parkin's RING0:RING2 or REP:RING1 interactions, two key hydrophobic interactions that maintain parkin's inhibited state (Trempe et al., 2013). Future structural and functional studies examining BAG5-parkin interaction and the effect of BAG5 on parkin with activating mutations could be undertaken to investigate these possibilities.

Crystallization of solution containing recombinant parkin and BAG5 followed by X-ray crystallography could be used to determine the true nature of the BAG5-parkin interaction. If crystallization proves challenging, or to complement a successful crystal structure, inferences on the interaction can be drawn from BAG5's effect on the recruitment of activated mutants. Tang et al. (2017) have designed parkin constructs that have mutations in key hydrophobic residues to abolish interactions at the RING0:RING2 interface (F146A), the Ubl:RING1 interface (N273K), and the REP:RING1 interface (W403A). All of these activating mutations accelerate parkin

recruitment kinetics (Tang et al., 2017). Exploring BAG5's ability to regulate these activated mutants would provide support for the hypothesis that BAG5 stabilizes parkin's autoinhibited state. If BAG5 does indeed stabilize the RING0:RING2, but not Ubl:RING1 or REP:RING1 interfaces, it would be expected to still impair the recruitment of N273K and W403A, but not F146A mutants that already have the RING0:RING2 interface released. If BAG5 stabilizes the REP:RING1, but not RING0:RING2 and Ubl:RING1 interfaces, it would be expected to impair F146A and N273K, but not W403A mutants. If BAG5 stabilizes both RING0:RING2 and REP:RING1 interfaces, it should impair recruitment of N273K, and to a lesser degree, impair both F146A and W403A single mutants, but not F146A/W403A double mutant parkin. The fact that BAG5 interacts with both the regions of parkin that contains RING0 and RING2 suggest that stabilization of the RING0:RING2 interface is likely the more important interaction.

Taken together our findings indicate that BAG5 impairs PINK1 and parkin directed mitophagy by delaying parkin recruitment to the mitochondria. Loss of PINK1 or parkin function leads to early onset parkinsonism (Kitada et al., 1998; Valente et al., 2004), so BAG5's impairment of one of their primary functions highlights its potential role in the pathobiology of PD. Furthermore, BAG5 is known have a generally negative regulatory effect on several other parkinsonian gene products. BAG5 acts in a complex with LRRK2 to enhance pathological clearance of the trans-Golgi network (Beilina et al., 2014). It also prevents DJ-1's protection against oxidative stress by preventing its dimerization and translocation to mitochondria (Qin et al., 2017). BAG5 indirectly affects α-synuclein toxicity by inhibiting its ubiquitination oligomers by CHIP, and inhibiting Hsp70, which can mitigate α-synuclein toxicity (Kalia et al., 2011; Kalia et al., 2004). Considering these effects together, it is tempting to speculate that BAG5 is a node that links together the diverse protein networks that are related to Parkinson's disease, and for

that reason, may represent a good therapeutic target. The contexts in which BAG5 is shown to enhance neurodegeneration, however, are limited to acute models of PD. Further study into the role of BAG5 in rodent or non-human primate models that better recapitulate the chronic features of PD by overexpressing mutant α-synuclein species will be necessary to investigate the hypothesis that BAG5 is a valid therapeutic target in PD.

References

Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Allen, G.F., Toth, R., James, J., and Ganley, I.G. (2013). Loss of iron triggers PINK1/Parkin-independent mitophagy. EMBO reports *14*, 1127-1135.

Annesi, G., Savettieri, G., Pugliese, P., D'Amelio, M., Tarantino, P., Ragonese, P., La Bella, V., Piccoli, T., Civitelli, D., and Annesi, F. (2005). DJ-1 mutations and parkinsonism-dementia-amyotrophic lateral sclerosis complex. Annals of neurology *58*, 803-807.

Arakawa, A., Handa, N., Ohsawa, N., Shida, M., Kigawa, T., Hayashi, F., Shirouzu, M., and Yokoyama, S. (2010). The C-terminal BAG domain of BAG5 induces conformational changes of the Hsp70 nucleotide-binding domain for ADP-ATP exchange. Structure *18*, 309-319.

Arndt, V., Daniel, C., Nastainczyk, W., Alberti, S., and Höhfeld, J. (2005). BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP. Molecular biology of the cell *16*, 5891-5900.

Ashrafi, G., Schlehe, J.S., LaVoie, M.J., and Schwarz, T.L. (2014). Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. The Journal of Cell Biology *206*, 655-670.

Auluck, P.K., Chan, H.Y., Trojanowski, J.Q., Lee, V.M., and Bonini, N.M. (2002). Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. Science *295*, 865-868.

Behl, C. (2016). Breaking BAG: The Co-Chaperone BAG3 in Health and Disease. Trends in pharmacological sciences.

Beilina, A., Rudenko, I.N., Kaganovich, A., Civiero, L., Chau, H., Kalia, S.K., Kalia, L.V., Lobbestael, E., Chia, R., and Ndukwe, K. (2014). Unbiased screen for interactors of leucine-rich repeat kinase 2 supports a common pathway for sporadic and familial Parkinson disease. Proceedings of the National Academy of Sciences *111*, 2626-2631.

Bender, A., Krishnan, K.J., Morris, C.M., Taylor, G.A., Reeve, A.K., Perry, R.H., Jaros, E., Hersheson, J.S., Betts, J., and Klopstock, T. (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nature genetics *38*, 515-517.

Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., and Greenamyre, J.T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nature neuroscience *3*, 1301-1306.

Bian, M., Liu, J., Hong, X., Yu, M., Huang, Y., Sheng, Z., Fei, J., and Huang, F. (2012). Overexpression of parkin ameliorates dopaminergic neurodegeneration induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine in mice. PloS one *7*, e39953.

Bingol, B., Tea, J.S., Phu, L., Reichelt, M., Bakalarski, C.E., Song, Q., Foreman, O., Kirkpatrick, D.S., and Sheng, M. (2014). The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. Nature *510*, 370-375.

Bonifati, V., Rizzu, P., Van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., and Joosse, M. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science *299*, 256-259.

Bratic, A., and Larsson, N.-G. (2013). The role of mitochondria in aging. The Journal of clinical investigation *123*, 951.

Brecknell, J., Dunnett, S., and Fawcett, J. (1995). A quantitative study of cell death in the substantia nigra following a mechanical lesion of the medial forebrain bundle. Neuroscience *64*, 219-227.

Briknarová, K., Takayama, S., Homma, S., Baker, K., Cabezas, E., Hoyt, D.W., Li, Z., Satterthwait, A.C., and Ely, K.R. (2002). BAG4/SODD protein contains a short BAG domain. Journal of Biological Chemistry *277*, 31172-31178.

Bukau, B., and Horwich, A.L. (1998). The Hsp70 and Hsp60 chaperone machines. Cell *92*, 351-366.

Burman, J.L., Pickles, S., Wang, C., Sekine, S., Vargas, J.N.S., Zhang, Z., Youle, A.M., Nezich, C.L., Wu, X., and Hammer, J.A. (2017). Mitochondrial fission facilitates the selective mitophagy of protein aggregates. J Cell Biol, jcb. 201612106.

Cai, Q., Zakaria, H.M., Simone, A., and Sheng, Z.H. (2012). Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. Current biology : CB *22*, 545-552.

Carroll, R.G., Hollville, E., and Martin, S.J. (2014). Parkin sensitizes toward apoptosis induced by mitochondrial depolarization through promoting degradation of Mcl-1. Cell reports *9*, 1538-1553.

Cartoni, R., Norsworthy, M.W., Bei, F., Wang, C., Li, S., Zhang, Y., Gabel, C.V., Schwarz, T.L., and He, Z. (2016). The mammalian-specific protein Armcx1 regulates mitochondrial transport during axon regeneration. Neuron *92*, 1294-1307.

Chan, N.C., Salazar, A.M., Pham, A.H., Sweredoski, M.J., Kolawa, N.J., Graham, R.L.J., Hess, S., and Chan, D.C. (2011). Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. Human molecular genetics, ddr048.

Chan, P., DeLanney, L.E., Irwin, I., Langston, J.W., and Di Monte, D. (1991). Rapid ATP loss caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mouse brain. J Neurochem *57*, 348-351.

Charan, R., Johnson, B., Zaganelli, S., Nardozzi, J., and LaVoie, M. (2014). Inhibition of apoptotic Bax translocation to the mitochondria is a central function of parkin. Cell death & disease *5*, e1313.

Che, X.-Q., Tang, B.-S., Wang, H.-F., Yan, X.-X., Jiang, H., Shen, L., Xu, Q., Wang, G.-H., Zhang, H.-N., and Wang, C.-Y. (2015). The BAG2 and BAG5 proteins inhibit the ubiquitination of pathogenic ataxin3-80Q. International Journal of Neuroscience *125*, 390-394.

Chinta, S.J., Mallajosyula, J.K., Rane, A., and Andersen, J.K. (2010). Mitochondrial alpha-synuclein accumulation impairs complex I function in dopaminergic neurons and results in increased mitophagy in vivo. Neuroscience letters *486*, 235-239.

Cicchetti, F., Lapointe, N., Roberge-Tremblay, A., Saint-Pierre, M., Jimenez, L., Ficke, B.W., and Gross, R.E. (2005). Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. Neurobiology of Disease *20*, 360-371.

Ciechanover, A., and Kwon, Y.T. (2017). Protein Quality Control by Molecular Chaperones in Neurodegeneration. Frontiers in Neuroscience *11*.

Clark, I.E., Dodson, M.W., Jiang, C., Cao, J.H., Huh, J.R., Seol, J.H., Yoo, S.J., Hay, B.A., and Guo, M. (2006). Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature *441*, 1162-1166.

Cornelissen, T., Haddad, D., Wauters, F., Van Humbeeck, C., Mandemakers, W., Koentjoro, B., Sue, C., Gevaert, K., De Strooper, B., and Verstreken, P. (2014). The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy. Human molecular genetics *23*, 5227-5242.

Cuervo, A.M., Stefanis, L., Fredenburg, R., Lansbury, P.T., and Sulzer, D. (2004). Impaired degradation of mutant α-synuclein by chaperone-mediated autophagy. Science *305*, 1292-1295.

Dauer, W., Kholodilov, N., Vila, M., Trillat, A.-C., Goodchild, R., Larsen, K.E., Staal, R., Tieu, K., Schmitz, Y., and Yuan, C.A. (2002). Resistance of α-synuclein null mice to the parkinsonian neurotoxin MPTP. Proceedings of the National Academy of Sciences *99*, 14524-14529.

Deas, E., Plun-Favreau, H., Gandhi, S., Desmond, H., Kjaer, S., Loh, S.H.Y., Renton, A.E.M., Harvey, R.J., Whitworth, A.J., and Martins, L.M. (2011). PINK1 cleavage at position A103 by the mitochondrial protease PARL. Human molecular genetics *20*, 867-879.

Devi, L., Raghavendran, V., Prabhu, B.M., Avadhani, N.G., and Anandatheerthavarada, H.K. (2008). Mitochondrial import and accumulation of α-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. Journal of Biological Chemistry *283*, 9089-9100.

Di Maio, R., Barrett, P.J., Hoffman, E.K., Barrett, C.W., Zharikov, A., Borah, A., Hu, X., McCoy, J., Chu, C.T., and Burton, E.A. (2016). α-Synuclein binds to TOM20 and inhibits mitochondrial protein import in Parkinson's disease. Science translational medicine *8*, 342ra378-342ra378.

Doherty, K.M., Silveira-Moriyama, L., Parkkinen, L., and et al. (2013). Parkin disease: A clinicopathologic entity? JAMA Neurology *70*, 571-579.

Durcan, T.M., Tang, M.Y., Pérusse, J.R., Dashti, E.A., Aguileta, M.A., McLelland, G.L., Gros, P., Shaler, T.A., Faubert, D., and Coulombe, B. (2014). USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. The EMBO journal, e201489729.

Espay, A.J., Brundin, P., and Lang, A.E. (2017). Precision medicine for disease modification in Parkinson disease. Nat Rev Neurol *13*, 119-126.

Ferreira, M., and Massano, J. (2017). An updated review of Parkinson's disease genetics and clinicopathological correlations. Acta Neurologica Scandinavica *135*, 273-284.

Fiesel, F.C., Ando, M., Hudec, R., Hill, A.R., Castanedes-Casey, M., Caulfield, T.R., Moussaud-Lamodière, E.L., Stankowski, J.N., Bauer, P.O., and Lorenzo-Betancor, O. (2015). (Patho-) physiological relevance of PINK1-dependent ubiquitin phosphorylation. EMBO reports *16*, 1114-1130.

Flower, T.R., Chesnokova, L.S., Froelich, C.A., Dixon, C., and Witt, S.N. (2005). Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease. Journal of molecular biology *351*, 1081-1100.

Fox, S.H., and Brotchie, J.M. (2010). Chapter 7 - The MPTP-lesioned non-human primate models of Parkinson's disease. Past, present, and future. In Progress in Brain Research, A. Björklund, and M.A. Cenci, eds. (Elsevier), pp. 133-157.

Friesen, E.L., De Snoo, M.L., Rajendran, L., Kalia, L.V., and Kalia, S.K. (2017). Chaperone-Based Therapies for Disease Modification in Parkinson's Disease. Parkinson's Disease *2017*, 11.

Gandhi, S., Wood-Kaczmar, A., Yao, Z., Plun-Favreau, H., Deas, E., Klupsch, K., Downward, J., Latchman, D.S., Tabrizi, S.J., and Wood, N.W. (2009). PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. Molecular cell *33*, 627-638.

Gautier, C.A., Giaime, E., Caballero, E., Núñez, L., Song, Z., Chan, D., Villalobos, C., and Shen, J. (2012). Regulation of mitochondrial permeability transition pore by PINK1. Molecular neurodegeneration *7*, 22.

Gautier, C.A., Kitada, T., and Shen, J. (2008). Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proceedings of the National Academy of Sciences *105*, 11364-11369.

Gladkova, C., Schubert, A.F., Wagstaff, J.L., Pruneda, J.N., Freund, S.M., and Komander, D. (2017). An invisible ubiquitin conformation is required for efficient phosphorylation by PINK1. The EMBO Journal, e201797876.

Goldberg, M.S., Fleming, S.M., Palacino, J.J., Cepeda, C., Lam, H.A., Bhatnagar, A., Meloni, E.G., Wu, N., Ackerson, L.C., and Klapstein, G.J. (2003). Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. Journal of Biological Chemistry *278*, 43628-43635.

Green, D.R., Galluzzi, L., and Kroemer, G. (2011). Mitochondria and the autophagy–inflammation–cell death axis in organismal aging. Science *333*, 1109-1112.

Greene, A.W., Grenier, K., Aguileta, M.A., Muise, S., Farazifard, R., Haque, M.E., McBride, H.M., Park, D.S., and Fon, E.A. (2012). Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. EMBO reports *13*, 378-385.

Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M.B., and Pallanck, L.J. (2003). Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proceedings of the National Academy of Sciences *100*, 4078-4083.

Guzman, J.N., Sanchez-Padilla, J., Wokosin, D., Kondapalli, J., Ilijic, E., Schumacker, P.T., and Surmeier, D.J. (2010). Oxidant stress evoked by pacemaking in dopaminergic neurons is attenuated by DJ-1. Nature *468*, 696.

Han, S.M., Baig, H.S., and Hammarlund, M. (2016). Mitochondria localize to injured axons to support regeneration. Neuron *92*, 1308-1323.

Haque, M.E., Thomas, K.J., D'Souza, C., Callaghan, S., Kitada, T., Slack, R.S., Fraser, P., Cookson, M.R., Tandon, A., and Park, D.S. (2008). Cytoplasmic Pink1 activity protects neurons from dopaminergic neurotoxin MPTP. Proceedings of the National Academy of Sciences *105*, 1716-1721.

Hasson, S.A., Kane, L.A., Yamano, K., Huang, C.-H., Sliter, D.A., Buehler, E., Wang, C., Heman-Ackah, S.M., Hessa, T., and Guha, R. (2013). High-content genome-wide RNAi screens identify regulators of parkin upstream of mitophagy. Nature *504*, 291-295.

Healy, D.G., Falchi, M., O'Sullivan, S.S., Bonifati, V., Durr, A., Bressman, S., Brice, A., Aasly, J., Zabetian, C.P., Goldwurm, S.*, et al.* (2008). Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. The Lancet Neurology *7*, 583-590.

Heeman, B., Van den Haute, C., Aelvoet, S.-A., Valsecchi, F., Rodenburg, R.J., Reumers, V., Debyser, Z., Callewaert, G., Koopman, W.J., and Willems, P.H. (2011). Depletion of PINK1 affects mitochondrial metabolism, calcium homeostasis and energy maintenance. J Cell Sci *124*, 1115-1125.

Heikkila, R.E., Manzino, L., Cabbat, F.S., and Duvoisin, R.C. (1984). Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. Nature *311*, 467-469.

Höhfeld, J., and Jentsch, S. (1997). GrpE-like regulation of the Hsc70 chaperone by the anti-apoptotic protein BAG-1. The EMBO journal *16*, 6209-6216.

Hollville, E., Carroll, R.G., Cullen, S.P., and Martin, S.J. (2014). Bcl-2 family proteins participate in mitochondrial quality control by regulating Parkin/PINK1-dependent mitophagy. Molecular cell *55*, 451-466.

Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L., and Conlon, P.J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. Nature Biotechnology *6*, 1204-1210.

Hristova, V.A., Beasley, S.A., Rylett, R.J., and Shaw, G.S. (2009). Identification of a novel Zn2+-binding domain in the autosomal recessive juvenile Parkinson-related E3 ligase parkin. Journal of Biological Chemistry *284*, 14978-14986.

Hu, Z.-y., Chen, B., Zhang, J.-p., and Ma, Y.-y. (2017). Up-regulation of autophagy-related gene 5 (ATG5) protects dopaminergic neurons in a zebrafish model of Parkinson's disease. Journal of Biological Chemistry *292*, 18062-18074.

Ibanez, P., Bonnet, A., Debarges, B., Lohmann, E., Tison, F., Agid, Y., Dürr, A., Brice, A., Pollak, P., and Group, F.P.s.D.G.S. (2004). Causal relation between α-synuclein locus duplication as a cause of familial Parkinson's disease. The Lancet *364*, 1169-1171.

Ivatt, R.M., Sanchez-Martinez, A., Godena, V.K., Brown, S., Ziviani, E., and Whitworth, A.J. (2014). Genome-wide RNAi screen identifies the Parkinson disease GWAS risk locus SREBF1 as a regulator of mitophagy. Proceedings of the National Academy of Sciences of the United States of America *111*, 8494-8499.

Jin, S.M., Lazarou, M., Wang, C., Kane, L.A., Narendra, D.P., and Youle, R.J. (2010). Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. The Journal of cell biology *191*, 933-942.

Jin, S.M., and Youle, R.J. (2013). The accumulation of misfolded proteins in the mitochondrial matrix is sensed by PINK1 to induce PARK2/Parkin-mediated mitophagy of polarized mitochondria. Autophagy *9*, 1750-1757.

Johnson, B.N., Berger, A.K., Cortese, G.P., and LaVoie, M.J. (2012). The ubiquitin E3 ligase parkin regulates the proapoptotic function of Bax. Proceedings of the National Academy of Sciences *109*, 6283-6288.

Kalia, L.V., Kalia, S.K., Chau, H., Lozano, A.M., Hyman, B.T., and McLean, P.J. (2011). Ubiquitinylation of α-synuclein by carboxyl terminus Hsp70-interacting protein (CHIP) is regulated by Bcl-2-associated athanogene 5 (BAG5). PloS one *6*, e14695.

Kalia, L.V., Kalia, S.K., McLean, P.J., Lozano, A.M., and Lang, A.E. (2013). α-Synuclein oligomers and clinical implications for Parkinson disease. Annals of neurology *73*, 155-169.

Kalia, L.V., and Lang, A.E. (2015). Parkinson's disease. The Lancet *386*, 896-912.

Kalia, S.K., Lee, S., Smith, P.D., Liu, L., Crocker, S.J., Thorarinsdottir, T.E., Glover, J.R., Fon, E.A., Park, D.S., and Lozano, A.M. (2004). BAG5 inhibits parkin and enhances dopaminergic neuron degeneration. Neuron *44*, 931-945.

Kampinga, H.H., Hageman, J., Vos, M.J., Kubota, H., Tanguay, R.M., Bruford, E.A., Cheetham, M.E., Chen, B., and Hightower, L.E. (2009). Guidelines for the nomenclature of the human heat shock proteins. Cell Stress and Chaperones *14*, 105-111.

Kane, L.A., Lazarou, M., Fogel, A.I., Li, Y., Yamano, K., Sarraf, S.A., Banerjee, S., and Youle, R.J. (2014). PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. The Journal of Cell Biology *205*, 143-153.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer research *51*, 6304-6311.

Katayama, H., Kogure, T., Mizushima, N., Yoshimori, T., and Miyawaki, A. (2011). A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. Chemistry & biology *18*, 1042-1052.

Kaushik, S., and Cuervo, A.M. (2012). Chaperone-mediated autophagy: a unique way to enter the lysosome world. Trends in cell biology *22*, 407-417.

Kieburtz, K., Tilley, B.C., Elm, J.J., Babcock, D., Hauser, R., Ross, G.W., Augustine, A.H., Augustine, E.U., Aminoff, M.J., and Bodis-Wollner, I.G. (2015). Effect of creatine monohydrate on clinical progression in patients with Parkinson disease: a randomized clinical trial. Jama *313*, 584-593.

Kim, R.H., Smith, P.D., Aleyasin, H., Hayley, S., Mount, M.P., Pownall, S., Wakeham, A., You-Ten, A.J., Kalia, S.K., and Horne, P. (2005). Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyrindine (MPTP) and oxidative stress. Proceedings of the National Academy of Sciences of the United States of America *102*, 5215-5220.

Kinnally, K.W., Peixoto, P.M., Ryu, S.-Y., and Dejean, L.M. (2011). Is mPTP the gatekeeper for necrosis, apoptosis, or both? Biochimica et Biophysica Acta (BBA)-Molecular Cell Research *1813*, 616-622.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature *392*, 605-608.

Kitada, T., Pisani, A., Porter, D.R., Yamaguchi, H., Tscherter, A., Martella, G., Bonsi, P., Zhang, C., Pothos, E.N., and Shen, J. (2007). Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. Proceedings of the National Academy of Sciences *104*, 11441-11446.

Kitada, T., Tong, Y., Gautier, C.A., and Shen, J. (2009). Absence of nigral degeneration in aged parkin/DJ-1/PINK1 triple knockout mice. J Neurochem *111*, 696-702.

Kitayama, S., Wang, J.B., and Uhl, G.R. (1993). Dopamine transporter mutants selectively enhance MPP+ transport. Synapse *15*, 58-62.

Klucken, J., Shin, Y., Hyman, B.T., and McLean, P.J. (2004a). A single amino acid substitution differentiates Hsp70-dependent effects on alpha-synuclein degradation and toxicity. Biochem Biophys Res Commun *325*, 367-373.

Klucken, J., Shin, Y., Masliah, E., Hyman, B.T., and McLean, P.J. (2004b). Hsp70 reduces α-synuclein aggregation and toxicity. Journal of Biological Chemistry *279*, 25497-25502.

Kondapalli, C., Kazlauskaite, A., Zhang, N., Woodroof, H.I., Campbell, D.G., Gourlay, R., Burchell, L., Walden, H., Macartney, T.J., Deak, M.*, et al.* (2012). PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biology *2*.

Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., Kimura, Y., Tsuchiya, H., Yoshihara, H., Hirokawa, T.*, et al.* (2014). Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature *510*.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet *18*, 106-108.

Kujoth, G., Hiona, A., Pugh, T., Someya, S., Panzer, K., Wohlgemuth, S., Hofer, T., Seo, A., Sullivan, R., and Jobling, W. (2005). Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science *309*, 481-484.

Kumar, A., Tamjar, J., Waddell, A.D., Woodroof, H.I., Raimi, O.G., Shaw, A.M., Peggie, M., Muqit, M.M., and van Aalten, D.M. (2017). Structure of PINK1 and mechanisms of Parkinson's disease-associated mutations. eLife *6*.

Langston, J., Ballard, P., Tetrud, J., and Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science *219*, 979-980.

Lazarou, M., Sliter, D.A., Kane, L.A., Sarraf, S.A., Wang, C., Burman, J.L., Sideris, D.P., Fogel, A.I., and Youle, R.J. (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature *524*, 309-314.

Lee, Y., Stevens, D.A., Kang, S.-U., Jiang, H., Lee, Y.-I., Ko, H.S., Scarffe, L.A., Umanah, G.E., Kang, H., and Ham, S. (2017). PINK1 primes parkin-mediated ubiquitination of PARIS in dopaminergic neuronal survival. Cell reports *18*, 918-932.

Lefebvre, V., Du, Q., Baird, S., Ng, A.C., Nascimento, M., Campanella, M., McBride, H.M., and Screaton, R.A. (2013). Genome-wide RNAi screen identifies ATPase inhibitory factor 1 (ATPIF1) as essential for PARK2 recruitment and mitophagy. Autophagy *9*, 1770-1779.

Lesage, S., and Brice, A. (2009). Parkinson's disease: from monogenic forms to genetic susceptibility factors. Human Molecular Genetics *18*, R48-R59.

Lin, M.T., and Beal, M.F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature *443*, 787-795.

Lin, M.T., Cantuti-Castelvetri, I., Zheng, K., Jackson, K.E., Tan, Y.B., Arzberger, T., Lees, A.J., Betensky, R.A., Beal, M.F., and Simon, D.K. (2012). Somatic mitochondrial DNA mutations in early Parkinson's and incidental Lewy body disease. Annals of Neurology *71*, 850-854.

Liu, G., Zhang, C., Yin, J., Li, X., Cheng, F., Li, Y., Yang, H., Uéda, K., Chan, P., and Yu, S. (2009). α-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity. Neuroscience Letters *454*, 187-192.

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Lücking , C.B., Dürr , A., Bonifati , V., Vaughan , J., De Michele , G., Gasser , T., Harhangi , B.S., Meco , G., Denèfle , P., Wood , N.W.*, et al.* (2000). Association between Early-Onset Parkinson's Disease and Mutations in the Parkin Gene. New England Journal of Medicine *342*, 1560-1567.

Ludtmann, M.H., Angelova, P.R., Ninkina, N.N., Gandhi, S., Buchman, V.L., and Abramov, A.Y. (2016). Monomeric alpha-synuclein exerts a physiological role on brain ATP synthase. Journal of Neuroscience *36*, 10510-10521.

Luth, E.S., Stavrovskaya, I.G., Bartels, T., Kristal, B.S., and Selkoe, D.J. (2014). Soluble, prefibrillar α-synuclein oligomers promote complex I-dependent, Ca2+-induced mitochondrial dysfunction. Journal of Biological Chemistry *289*, 21490-21507.

Mak, S.K., McCormack, A.L., Manning-Boğ, A.B., Cuervo, A.M., and Di Monte, D.A. (2010). Lysosomal degradation of α-synuclein in vivo. Journal of Biological Chemistry *285*, 13621-13629.

Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C.A., Sou, Y.-s., Saiki, S., Kawajiri, S., Sato, F.*, et al.* (2010). PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. The Journal of Cell Biology *189*, 211-221.

McCoy, M.K., Kaganovich, A., Rudenko, I.N., Ding, J., and Cookson, M.R. (2014). Hexokinase activity is required for recruitment of parkin to depolarized mitochondria. Human Molecular Genetics *23*, 145-156.

McLean, P.J., Kawamata, H., Shariff, S., Hewett, J., Sharma, N., Ueda, K., Breakefield, X.O., and Hyman, B.T. (2002). TorsinA and heat shock proteins act as molecular chaperones: suppression of α-synuclein aggregation. Journal of neurochemistry *83*, 846-854.

McLelland, G.-L., Lee, S.A., McBride, H.M., and Fon, E.A. (2016). Syntaxin-17 delivers PINK1/parkin-dependent mitochondrial vesicles to the endolysosomal system. J Cell Biol *214*, 275-291.

McLelland, G.L., Soubannier, V., Chen, C.X., McBride, H.M., and Fon, E.A. (2014). Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. The EMBO journal *33*, 282-295.

McWilliams, T.G., Prescott, A.R., Allen, G.F., Tamjar, J., Munson, M.J., Thomson, C., Muqit, M.M., and Ganley, I.G. (2016). mito-QC illuminates mitophagy and mitochondrial architecture in vivo. J Cell Biol, jcb. 201603039.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., and Cyr, D.M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nature cell biology *3*, 100-105.

Moloney, T.C., Hyland, R., O'Toole, D., Paucard, A., Kirik, D., O'Doherty, A., Gorman, A.M., and Dowd, E. (2014). Heat Shock Protein 70 Reduces α-Synuclein-Induced Predegenerative Neuronal Dystrophy in the α-Synuclein Viral Gene Transfer Rat Model of Parkinson's Disease. CNS neuroscience & therapeutics *20*, 50-58.

Müller-Rischart, A.K., Pilsl, A., Beaudette, P., Patra, M., Hadian, K., Funke, M., Peis, R., Deinlein, A., Schweimer, C., and Kuhn, P.-H. (2013). The E3 ligase parkin maintains mitochondrial integrity by increasing linear ubiquitination of NEMO. Molecular cell *49*, 908-921.

Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO reports *2*, 1133-1138.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochemical Journal *417*, 1-13.

Narendra, D., Kane, L.A., Hauser, D.N., Fearnley, I.M., and Youle, R.J. (2010a). p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy *6*, 1090-1106.

Narendra, D., Tanaka, A., Suen, D.-F., and Youle, R.J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. The Journal of cell biology *183*, 795-803.

Narendra, D.P., Jin, S.M., Tanaka, A., Suen, D.-F., Gautier, C.A., Shen, J., Cookson, M.R., and Youle, R.J. (2010b). PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol *8*, e1000298.

Noyce, A.J., Bestwick, J.P., Silveira-Moriyama, L., Hawkes, C.H., Giovannoni, G., Lees, A.J., and Schrag, A. (2012). Meta-analysis of early nonmotor features and risk factors for Parkinson disease. Annals of neurology *72*, 893-901.

Nuytemans, K., Theuns, J., Cruts, M., and Van Broeckhoven, C. (2010). Genetic Etiology of Parkinson Disease Associated with Mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 Genes: A Mutation Update. Human Mutation *31*, 763-780.

Okatsu, K., Koyano, F., Kimura, M., Kosako, H., Saeki, Y., Tanaka, K., and Matsuda, N. (2015). Phosphorylated ubiquitin chain is the genuine Parkin receptor. J Cell Biol, jcb. 201410050.

Okatsu, K., Saisho, K., Shimanuki, M., Nakada, K., Shitara, H., Sou, Y.s., Kimura, M., Sato, S., Hattori, N., and Komatsu, M. (2010). p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. Genes to Cells *15*, 887-900.

Ordureau, A., Sarraf, S.A., Duda, D.M., Heo, J.-M., Jedrychowski, M.P., Sviderskiy, V.O., Olszewski, J.L., Koerber, J.T., Xie, T., and Beausoleil, S.A. (2014). Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. Molecular cell *56*, 360-375.

Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., and Kim, J.-M. (2006). Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature *441*, 1157-1161.

Parkinson, J. (1817). An essay on the shaking palsy (Printed by Whittingham and Rowland for Sherwood, Neely, and Jones) (London).

Perez, F.A., and Palmiter, R.D. (2005). Parkin-deficient mice are not a robust model of parkinsonism. Proceedings of the National Academy of Sciences of the United States of America *102*, 2174-2179.

Periquet, M., Latouche, M., Lohmann, E., Rawal, N., De Michele, G., Ricard, S., Teive, H.l., Fraix, V.r., Vidailhet, M., Nicholl, D.*, et al.* (2003). Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain *126*, 1271-1278.

Pickrell, A.M., Huang, C.-H., Kennedy, S.R., Ordureau, A., Sideris, D.P., Hoekstra, J.G., Harper, J.W., and Youle, R.J. (2015). Endogenous Parkin preserves dopaminergic substantia nigral neurons following mitochondrial DNA mutagenic stress. Neuron *87*, 371-381.

Pickrell, A.M., and Youle, R.J. (2015). The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. Neuron *85*, 257-273.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., and Boyer, R. (1997). Mutation in the α-synuclein gene identified in families with Parkinson's disease. science *276*, 2045-2047.

Pringsheim, T., Jette, N., Frolkis, A., and Steeves, T.D.L. (2014). The prevalence of Parkinson's disease: A systematic review and meta-analysis. Movement Disorders *29*, 1583-1590.

Puschmann, A., Fiesel, F.C., Caulfield, T.R., Hudec, R., Ando, M., Truban, D., Hou, X., Ogaki, K., Heckman, M.G., James, E.D.*, et al.* (2017). Heterozygous PINK1 p.G411S increases risk of Parkinson's disease via a dominant-negative mechanism. Brain *140*, 98-117.

Qin, L.-x., Tan, J.-q., Zhang, H.-n., Rizwana, K., Lu, J.-h., Tang, J.-g., Jiang, B., Shen, X.-m., Guo, J.-f., and Tang, B.-s. (2017). BAG5 Interacts with DJ-1 and Inhibits the Neuroprotective Effects of DJ-1 to Combat Mitochondrial Oxidative Damage. Oxidative Medicine and Cellular Longevity *2017*.

Qu, D., Hage, A., Don-Carolis, K., Huang, E., Joselin, A., Safarpour, F., Marcogliese, P.C., Rousseaux, M.W.C., Hewitt, S.J., and Huang, T. (2015). BAG2 Gene-mediated Regulation of PINK1 Protein Is Critical for Mitochondrial Translocation of PARKIN and Neuronal Survival. Journal of Biological Chemistry *290*, 30441-30452.

Rakovic, A., Grünewald, A., Voges, L., Hofmann, S., Orolicki, S., Lohmann, K., and Klein, C. (2011). PINK1-interacting proteins: proteomic analysis of overexpressed PINK1. Parkinson's Disease *2011*.

Ramsay, R.R., Salach, J.I., and Singer, T.P. (1986). Uptake of the neurotoxin 1-methyl-4-phenylpyridine (MPP+) by mitochondria and its relation to the inhibition of the mitochondrial oxidation of NAD+-linked substrates by MPP+. Biochem Biophys Res Commun *134*, 743-748.

Ransom, B.R., Kunis, D.M., Irwin, I., and Langston, J.W. (1987). Astrocytes convert the parkinsonism inducing neurotoxin, MPTP, to its active metabolite, MPP+. Neurosci Lett *75*, 323-328.

Riachi, N.J., Harik, S.I., Kalaria, R.N., and Sayre, L.M. (1988). On the mechanisms underlying 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. II. Susceptibility among mammalian species correlates with the toxin's metabolic patterns in brain microvessels and liver. The Journal of pharmacology and experimental therapeutics *244*, 443-448.

Riley, B.E., Lougheed, J.C., Callaway, K., Velasquez, M., Brecht, E., Nguyen, L., Shaler, T., Walker, D., Yang, Y., Regnstrom, K.*, et al.* (2013). Structure and function of Parkin E3 ubiquitin ligase reveals aspects of RING and HECT ligases. Nature Communications *4*, 1982.

Sarraf, S.A., Raman, M., Guarani-Pereira, V., Sowa, M.E., Huttlin, E.L., Gygi, S.P., and Harper, J.W. (2013). Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. Nature *496*, 372-376.

Schapira, A.H., Cooper, J.M., Dexter, D., Clark, J.B., Jenner, P., and Marsden, C.D. (1990). Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem *54*, 823-827.

Schubert, A.F., Gladkova, C., Pardon, E., Wagstaff, J.L., Freund, S.M., Steyaert, J., Maslen, S.L., and Komander, D. (2017). Structure of PINK1 in complex with its substrate ubiquitin. Nature.

Seibler, P., Graziotto, J., Jeong, H., Simunovic, F., Klein, C., and Krainc, D. (2011). Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 iPS cells. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 5970-5976.

Sharma, N., Hewett, J., Ozelius, L.J., Ramesh, V., McLean, P.J., Breakefield, X.O., and Hyman, B.T. (2001). A close association of torsinA and α-synuclein in Lewy bodies: A fluorescence resonance energy transfer study. The American journal of pathology *159*, 339-344.

Shashidharan, P., Good, P., Hsu, A., Perl, D., Brin, M., and Olanow, C. (2000). TorsinA accumulation in Lewy bodies in sporadic Parkinson’s disease. Brain research *877*, 379-381.

Shen, R.S., Abell, C.W., Gessner, W., and Brossi, A. (1985). Serotonergic conversion of MPTP and dopaminergic accumulation of MPP+. FEBS letters *189*, 225-230.

Shimura, H., Hattori, N., Kubo, S.-i., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., and Tanaka, K. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nature genetics *25*, 302-305.

Shin, J.-H., Ko, H.S., Kang, H., Lee, Y., Lee, Y.-I., Pletinkova, O., Troconso, J.C., Dawson, V.L., and Dawson, T.M. (2011). PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in Parkinson's disease. Cell *144*, 689-702.

Shin, Y., Klucken, J., Patterson, C., Hyman, B.T., and McLean, P.J. (2005). The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates α-synuclein degradation decisions between proteasomal and lysosomal pathways. Journal of Biological Chemistry *280*, 23727-23734.

Sim, C.H., Gabriel, K., Mills, R.D., Culvenor, J.G., and Cheng, H.C. (2012). Analysis of the regulatory and catalytic domains of PTEN-induced kinase-1 (PINK1). Human mutation *33*, 1408-1422.

Singleton, A., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., and Nussbaum, R. (2003). α-Synuclein locus triplication causes Parkinson's disease. Science *302*, 841-841.

Smith, W.W., Pei, Z., Jiang, H., Dawson, V.L., Dawson, T.M., and Ross, C.A. (2006). Kinase activity of mutant LRRK2 mediates neuronal toxicity. Nature Neuroscience *9*, 1231.

Snow, B.J., Rolfe, F.L., Lockhart, M.M., Frampton, C.M., O'Sullivan, J.D., Fung, V., Smith, R.A., Murphy, M.P., and Taylor, K.M. (2010). A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson's disease. Movement Disorders *25*, 1670-1674.

Sondermann, H., Scheufler, C., Schneider, C., Höhfeld, J., Hartl, F.-U., and Moarefi, I. (2001). Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. Science *291*, 1553-1557.

Soubannier, V., Rippstein, P., Kaufman, B.A., Shoubridge, E.A., and McBride, H.M. (2012). Reconstitution of mitochondria derived vesicle formation demonstrates selective enrichment of oxidized cargo. PLoS One *7*, e52830.

Spillantini, M.G., Schmidt, M.L., Lee, V.M.-Y., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). α-Synuclein in Lewy bodies. Nature *388*, 839-840.

Stevens, D.A., Lee, Y., Kang, H.C., Lee, B.D., Lee, Y.-I., Bower, A., Jiang, H., Kang, S.-U., Andrabi, S.A., and Dawson, V.L. (2015). Parkin loss leads to PARIS-dependent declines in mitochondrial mass and respiration. Proceedings of the National Academy of Sciences *112*, 11696-11701.

Stolz, A., Ernst, A., and Dikic, I. (2014). Cargo recognition and trafficking in selective autophagy. Nature Cell Biology *16*, 495.

Sun, N., Yun, J., Liu, J., Malide, D., Liu, C., Rovira, I.I., Holmström, K.M., Fergusson, M.M., Yoo, Y.H., and Combs, C.A. (2015). Measuring in vivo mitophagy. Molecular cell *60*, 685-696.

Tait, S.W., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nature reviews Molecular cell biology *11*, 621-632.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Milian, J.A., and Reed, J.C. (1995). Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell *80*, 279-284.

Takayama, S., Xie, Z., and Reed, J.C. (1999). An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. Journal of Biological Chemistry *274*, 781-786.

Tang, M.Y., Vranas, M., Krahn, A.I., Pundlik, S., Trempe, J.-F., and Fon, E.A. (2017). Structure-guided mutagenesis reveals a hierarchical mechanism of Parkin activation. Nature Communications *8*.

Tanner, C.M., Kamel, F., Ross, G.W., Hoppin, J.A., Goldman, S.M., Korell, M., Marras, C., Bhudhikanok, G.S., Kasten, M., Chade, A.R.*, et al.* (2011). Rotenone, Paraquat, and Parkinson's Disease. Environmental Health Perspectives *119*, 866-872.

Tauchi, H., and Sato, T. (1968). Age changes in size and number of mitochondria of human hepatic cells. Journal of gerontology *23*, 454-461.

The Parkinson Study Group, Q.E.I. (2014). A randomized clinical trial of high-dosage coenzyme q10 in early parkinson disease: No evidence of benefit. JAMA Neurology *71*, 543-552.

The Parkinson Study Group, X. (1989). Effect of Deprenyl on the Progression of Disability in Early Parkinson's Disease. New England Journal of Medicine *321*, 1364-1371.

Tissiéres, A., Mitchell, H.K., and Tracy, U.M. (1974). Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. Journal of molecular biology *84*, 389IN9393-9392IN12398.

Trempe, J.-F., Sauvé, V., Grenier, K., Seirafi, M., Tang, M.Y., Ménade, M., Al-Abdul-Wahid, S., Krett, J., Wong, K., and Kozlov, G. (2013). Structure of parkin reveals mechanisms for ubiquitin ligase activation. Science *340*, 1451-1455.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly-y, M., Gidlöf, S., Oldfors, A., and Wibom, R. (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature *429*, 417-423.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., and Healy, D.G. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science *304*, 1158-1160.

Van Laar, V.S., Arnold, B., Cassady, S.J., Chu, C.T., Burton, E.A., and Berman, S.B. (2011). Bioenergetics of neurons inhibit the translocation response of Parkin following rapid mitochondrial depolarization. Human Molecular Genetics *20*, 927-940.

Venero, J.L., Revuelta, M., Cano, J., and Machado, A. (1997). Time course changes in the dopaminergic nigrostriatal system following transection of the medial forebrain bundle: detection of oxidatively modified proteins in substantia nigra. Journal of neurochemistry *68*, 2458-2468.

Vincow, E.S., Merrihew, G., Thomas, R.E., Shulman, N.J., Beyer, R.P., MacCoss, M.J., and Pallanck, L.J. (2013). The PINK1–Parkin pathway promotes both mitophagy and selective respiratory chain turnover in vivo. Proceedings of the National Academy of Sciences *110*, 6400-6405.

Violot, S., Carpentier, P., Blanchoin, L., and Bourgeois, D. (2009). Reverse pH-dependence of chromophore protonation explains the large Stokes shift of the red fluorescent protein mKeima. Journal of the American Chemical Society *131*, 10356-10357.

Wang, X., Guo, J., Fei, E., Mu, Y., He, S., Che, X., Tan, J., Xia, K., Zhang, Z., and Wang, G. (2014). BAG5 protects against mitochondrial oxidative damage through regulating PINK1 degradation. PloS one *9*, e86276.

Wang, Y., Serricchio, M., Jauregui, M., Shanbhag, R., Stoltz, T., Di Paolo, C.T., Kim, P.K., and McQuibban, G.A. (2015). Deubiquitinating enzymes regulate PARK2-mediated mitophagy. Autophagy *11*, 595-606.

Wauer, T., and Komander, D. (2013). Structure of the human Parkin ligase domain in an autoinhibited state. Embo j *32*, 2099-2112.

Wauer, T., Simicek, M., Schubert, A., and Komander, D. (2015). Mechanism of phospho-ubiquitin-induced PARKIN activation. Nature *524*, 370-374.

Wenzel, D.M., Lissounov, A., Brzovic, P.S., and Klevit, R.E. (2011). UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. Nature *474*, 105-108.

Woodroof, H.I., Pogson, J.H., Begley, M., Cantley, L.C., Deak, M., Campbell, D.G., van Aalten, D.M., Whitworth, A.J., Alessi, D.R., and Muqit, M.M. (2011). Discovery of catalytically active orthologues of the Parkinson's disease kinase PINK1: analysis of substrate specificity and impact of mutations. Open Biology *1*, 110012.

Xilouri, M., Brekk, O.R., Landeck, N., Pitychoutis, P.M., Papasilekas, T., Papadopoulou-Daifoti, Z., Kirik, D., and Stefanis, L. (2013). Boosting chaperone-mediated autophagy in vivo mitigates α-synuclein-induced neurodegeneration. Brain *136*, 2130-2146.

Xu, Z., Page, R.C., Gomes, M.M., Kohli, E., Nix, J.C., Herr, A.B., Patterson, C., and Misra, S. (2008). Structural basis of nucleotide exchange and client binding by the Hsp70 cochaperone Bag2. Nature structural & molecular biology *15*, 1309-1317.

Yamano, K., and Youle, R.J. (2013). PINK1 is degraded through the N-end rule pathway. Autophagy *9*, 1758-1769.

Yasuda, T., Hayakawa, H., Nihira, T., Ren, Y.-R., Nakata, Y., Nagai, M., Hattori, N., Miyake, K., Takada, M., and Shimada, T. (2011). Parkin-mediated protection of dopaminergic neurons in a chronic MPTP-minipump mouse model of Parkinson disease. Journal of Neuropathology & Experimental Neurology *70*, 686-697.

Yen, T.-C., Chen, Y.-S., King, K.-L., Yeh, S.-H., and Wei, Y.-H. (1989). Liver mitochondrial respiratory functions decline with age. Biochemical and biophysical research communications *165*, 994-1003.

Yokota, T., Sugawara, K., Ito, K., Takahashi, R., Ariga, H., and Mizusawa, H. (2003). Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. Biochemical and Biophysical Research Communications *312*, 1342-1348.

Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B.*, et al.* (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol *55*, 164-173.

Zhang, C., Lee, S., Peng, Y., Bunker, E., Giaime, E., Shen, J., Zhou, Z., and Liu, X. (2014). PINK1 triggers autocatalytic activation of Parkin to specify cell fate decisions. Current Biology *24*, 1854-1865.

Zhang, L., Shimoji, M., Thomas, B., Moore, D.J., Yu, S.-W., Marupudi, N.I., Torp, R., Torgner, I.A., Ottersen, O.P., and Dawson, T.M. (2005). Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. Human molecular genetics *14*, 2063-2073.

Zheng, Q., Huang, C., Guo, J., Tan, J., Wang, C., Tang, B., and Zhang, H. (2018). Hsp70 participates in PINK1-mediated mitophagy by regulating the stability of PINK1. Neuroscience Letters *662*, 264-270.