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Role of parkin in cytoprotection against misfolded SOD1 toxicity

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Doctor of Philosophy

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Abstract

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Amyotrophic lateral sclerosis (ALS) is a progressive neurological disease characterized by selective motor neuron death and accumulation of insoluble proteinaceous deposits in surviving motor neurons. Mutations in the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) cause ~20% of familial ALS cases. These mutations confer a toxic gain-of-function by inducing SOD1 misfolding and formation of cytotoxic SOD1 oligomers, which are sequestered into larger aggregates typical of ALS pathology. Misfolded proteins are normally efficiently handled by molecular chaperones and the ubiquitin-proteasome system, but when these protein quality control systems are impaired or overwhelmed, misfolded proteins can form soluble oligomers that are toxic to cells. The aggresome-autophagy pathway represents another cellular defense mechanism whereby cytotoxic oligomers are actively sequestered into a specialized type of perinuclear inclusion body known as the aggresome, where they are cleared by autophagy, a lysosome-dependent bulk degradation process. Although recent evidence indicates that misfolded SOD1 is targeted to perinuclear areas, forming aggresome-like inclusions, the mechanism responsible for this has not been identified. Our results show that parkin, an E3 ubiquitin-protein ligase linked to Parkinson disease, is a novel regulator of cellular defense against misfolded SOD1-induced cytotoxicity. We report that parkin mediates K63-linked polyubiquitination on mutant, but not wild-type, SOD1 and promotes misfolded SOD1 aggresome formation and clearance via the autophagy-lysosome system. Our findings reveal a novel role of parkin in cellular defense against misfolded SOD1 and suggests that targeting parkin may have therapeutic benefit for ALS.

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List of Abbreviations

3MA .....3-methyladenine (autophagy inhibitor)
AD..... Alzheimer disease
ALS .....amyotrophic lateral sclerosis
CMA ..... chaperone-mediated autophagy
CQ.....chloroquine (lysosome inhibitor)
DMSO..... dimethyl sulfoxide (vehicle)
DUB .....deubiquitinating enzyme
HD.....Huntington disease
KO.....knockout
LDH .....lactate dehydrogenase
MG132 ..... Z-Leu-Leu-Leu-al (proteasome inhibitor)
NMJ..... Neuromuscular junction
PD ..... Parkinson disease
PINK1 ..... PTEN-induced putative kinase 1
SOD1.....Cu/Zn superoxide dismutase
Ub.....ubiquitin
UPS .....ubiquitin-proteasome system
WT .....wild-type

Chapter 1: Introduction and Background

Opening Comments

Neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), Alzheimer disease (AD), and Huntington disease (HD) are characterized by progressive neuronal degeneration in specific regions of the nervous system. Pathologically, these diseases often include insoluble pathogenic protein deposits in nervous system tissues made of aggregated, misfolded disease-specific proteins. Treatments for these disorders to halt, slow, or even reverse progression is arguably one of the major medical challenges of our time, as current treatments are only palliative and unable to solve the root cause(s). Since aging is the greatest risk factor in developing neurodegenerative disease, and because of increased life expectancies, finding effective therapeutics is of considerable importance to relieve societal, economic, and healthcare burdens as the aging population increases. Therefore, insight into the underlying molecular mechanisms of these devastating diseases has important implications for future therapeutic strategies.

ALS is the most common adult-onset motor neuron disease, characterized by progressive paralysis and motor neuron death. Most ALS cases eventually lead to death within 5 years of onset (Haverkamp et al., 1995). An estimated 20% of genetic ALS is attributed to mutations in copper/zinc superoxide dismutase (SOD1) (Andersen, 2006; Rosen et al., 1993), an antioxidant enzyme that catalyzes the reduction of toxic superoxide radicals, which are a natural byproduct of some cellular processes. Mutant SOD1 aggregates are a pathological hallmark of ALS (Johnsson et al., 2008; Jonsson et al., 2004; Saccon et al., 2013) and may contribute to toxicity causing motor neuron death (Bruijn et al., 1998; Cleveland and Liu, 2000; Johnston et al., 2000). Mutant SOD1 has

been shown to localize to perinuclear aggregates reminiscent of aggresomes, as well as associate with K63-linked polyubiquitin (Tan et al., 2008) and markers of autophagy such as LC3-II (Li et al., 2008). However, the molecular machinery by which misfolded SOD1 is recognized and cleared from cells remains largely unknown.

The first portion of my dissertation work, described in Chapter 2, focuses primarily on the mechanism by which misfolded SOD1 is processed and cleared from cells. We found that the PD-linked E3 ubiquitin protein ligase parkin catalyzes K63-linked polyubiquitination specifically on mutant SOD1, and that mutant SOD1 undergoes autophagic degradation. This is the first report of SOD1 as a substrate of parkin ubiquitination, providing a novel association between these two proteins involved in neurodegenerative disease. Chapter 3 details our findings regarding *parkin* gene loss and mutant SOD1 in an animal model of ALS. In the *SOD1*^{G93A} mouse model, loss of parkin expression increases lifespan and delays onset of behavioral symptoms. Although these findings were unexpected, we propose that loss of parkin activates yet unidentified compensatory mechanisms that are protective towards motor neurons. The final chapter of this dissertation discusses the significance of our work, proposing future studies and commenting on the implications of these findings in furthering our understanding of the role of parkin and SOD1 in neurodegenerative disease.

1.1 The ubiquitin system and protein degradation

Components of ubiquitin signaling

Ubiquitination, a post-translational modification involving the attachment of ubiquitin to intracellular proteins, is a key regulatory mechanism required for many cellular functions. Ubiquitination occurs through a bond between the C terminus of ubiquitin and the ε-amino group of a substrate lysine residue. Several steps are required for this reaction, involving the coordinated action of three enzymes (Fig. 1.1A). First, a ubiquitin-activating enzyme (E1) forms a thioester linkage at its active site cysteine with the carboxyl group on the C terminus of ubiquitin (Ub), which activates ubiquitin for nucleophilic attack. Next, a ubiquitin-conjugating enzyme (E2) carries the activated ubiquitin, again forming a thioester linkage with ubiquitin at its active site cysteine, to a ubiquitin-protein ligase (E3) which catalyzes the transfer of ubiquitin from the E2 to a lysine on the substrate or other ubiquitin molecule. A monoubiquitinated substrate can then dissociate from the E3 or attain additional ubiquitins via multiple monoubiquitins or a polyubiquitin chain (Deshaies and Joazeiro, 2009; Pickart, 2001).

Ubiquitin, a 76-residue polypeptide, contains seven lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63) on which polyubiquitin chains can be formed via isopeptide bonds. Substrates can be monoubiquitinated or modified with a polyubiquitin chain, resulting in various cellular processing fates (Fig. 1.1B) (David et al., 2010; Pickart, 2001; Pickart and Eddins, 2004; Pickart and Fushman, 2004). Monoubiquitination mediates processes such as receptor endocytosis, DNA repair, and gene transcription (Chen and Sun, 2009; Sigismund et al., 2004; Sun and Chen, 2004). A polyubiquitin chain of at least 4 ubiquitins linked through K48 signals for the majority of

proteasomal degradation by the 26S proteasome, however, evidence also suggests that K6, K11, and K29 may also target substrates for the proteasome (Baboshina and Haas, 1996; Johnson et al., 1995; Shang et al., 2005). K63-linked polyubiquitin chains have been shown to play a role in the DNA damage response, as well as targeting substrates to the lysosome and the aggresome-autophagy pathway (Olzmann et al., 2007; Wen et al., 2006). K6-polyubiquitination has also been implicated in DNA repair (Pickart and Fushman, 2004), but overall the roles of K6, K27, K29, and K33 polyubiquitination are poorly understood (Pickart and Fushman, 2004; Sadowski et al., 2012).

E3s play an important role in determining the specificity of ubiquitination by pairing E2s and substrates. To illustrate the importance of E3 specificity, humans have only a few E1 enzymes and about 40 E2 enzymes with a conserved catalytic domain, while more than 500 E3 ligases have been described (Budhidarmo et al., 2012; Wenzel et al., 2011b). There are three major classes of E3s in eukaryotes, classified by conserved structural domains and the mechanism by which ubiquitination occurs: RING (really interesting new gene), HECT (homologous to E6-AP carboxy terminus), and RBR (RING-between-RING) (Berndsen and Wolberger, 2014).

RING E3s make up the majority of known E3s in humans (~400), while HECT-type E3s are much less prevalent (~20) (Budhidarmo et al., 2012). RING family E3s catalyze direct transfer of ubiquitin from the E2 enzyme onto the substrate protein and simultaneously bind both the E2-Ub thioester and the substrate (Budhidarmo et al., 2012). The RING domain of the E3 contacts a hydrophobic area on the donor ubiquitin, which immobilizes the ubiquitin and positions its C terminus in a groove on the E2 enzyme. Positioning the ubiquitin in this way places the reactive thioester between the

E2 and the ubiquitin C terminus in prime orientation to react with the acceptor lysine (Plechanovova et al., 2012). A transient bond between the E3 and the N-terminal helix of the associated E2 enzyme occurs while the ubiquitin is transferred onto the substrate protein (Berndsen and Wolberger, 2014). RING E3s typically contain multiple domains, including the E2-binding domain and domains that aid recruitment of substrates (Deshaies and Joazeiro, 2009). For example, the E3 gp78 contains a G2BR helical domain that binds to the E2 Ube2g2 on the face opposite the active site, triggering allosteric changes in Ube2g2 that open its active site for substrate binding and ubiquitination (Das et al., 2009). A subfamily of RING E3s known as cullin-RING E3s are multi-subunit proteins, containing cullin (a scaffold protein), a RING-box protein (containing a domain similar to RING domain), and an F-box protein (a bridging protein used for binding substrates) (Lydeard et al., 2013; Skaar et al., 2013). The majority of known cullin-RING E3s have not been studied in detail, but mutations or dysregulation in these proteins have been linked to several human diseases such as cancer, hypertension, and X-linked mental retardation (Boyden et al., 2012; Lee and Zhou, 2010; Lee and Zhou, 2012; Lydeard et al., 2013).

HECT and RBR family E3s ubiquitinate substrates using a two-step reaction where ubiquitin is transferred from the E2 to an active site cysteine in the E3, and then transferred from the E3 to the substrate. In HECT domain E3 ligases, a transthioesterification reaction first occurs where the ubiquitin is transferred from the E2 active site cysteine to a cysteine in the HECT domain. Next, the charged HECT-Ub thioester is attacked by a substrate lysine to transfer the ubiquitin onto the substrate (Berndsen and Wolberger, 2014). Structural studies utilizing Rsp5 of the Nedd4 family

of HECT E3s determined that the N lobe of the HECT domain binds the E2-Ub thioester and transfers the ubiquitin to the C lobe of the HECT domain, which contains the catalytic cysteine. The C lobe-Ub thioester then rotates to bring the donor ubiquitin in proximity to the substrate acceptor lysine (Kamadurai et al., 2013).

RBR domain E3 ligases share features of both RING and HECT E3s. Parkin, an E3 ligase linked to PD (Dawson and Dawson, 2010), was one of the first proteins in which an RBR domain was identified. The RBR domain is comprised of two RING domains, RING1 and RING2, which are separated by a conserved sequence known as the in-between-ring (IBR) domain. RING2 contains a catalytic cysteine that mediates ubiquitination in a HECT-like fashion, whereas RING1 recruits the associated E2 enzyme (Wenzel et al., 2011a).

Parkin ubiquitination proceeds in a two-step process. RING2 of parkin contains a catalytic triad of cysteine (conserved in all members of the RBR family), histidine, and an acidic residue (Seirafi et al., 2015). The first step is the formation of a ubiquitin-cysteine thioester where the ubiquitin C terminus is covalently bound to parkin's catalytic cysteine. Next, the ubiquitin is transferred from parkin to a substrate amino group (Seirafi et al., 2015), resulting in ubiquitination of the substrate protein. In parkin's compact autoinhibited state, the zinc-binding RING0 domain blocks the active site cysteine of RING2 and a significant conformational rearrangement would need to occur for the active site to be unmasked and bring RING1 (bound to the E2-Ub complex) to the RING2 active site (Berndsen and Wolberger, 2014). While the structural details of parkin activation are largely unknown, PINK1 (PTEN-induced putative kinase 1)-mediated phosphorylation has been described as a major method of parkin activation, at

least in mitophagy. When mitochondria are damaged, PINK1 phosphorylates parkin and recruits it to the depolarized mitochondria, where parkin ubiquitinates outer mitochondrial membrane proteins to promote mitophagy (Narendra et al., 2008).

Deubiquitinases (DUBs) play an important role in turnover of the ubiquitin pool by recognizing and removing ubiquitin (and in some cases, ubiquitin-like proteins) from substrate proteins and/or disassembling polyubiquitin chains (Heideker and Wertz, 2015). This process occurs by hydrolysis of the isopeptide bond between ubiquitin monomers or between ubiquitin and the substrate protein (Reyes-Turcu et al., 2009). The human genome encodes about 100 DUBs with specificity for ubiquitin, grouped into five major families: the ubiquitin C-terminal hydrolase (UCH), the ubiquitin specific protease (USP/UBP), the ovarian tumor (OTU), the Josephin domain containing, and the JAB1/MPN/Mov34 metalloenzyme (JAMM) domain containing families (Reyes-Turcu et al., 2009). The first four families are cysteine proteases, while the JAMM family members are zinc-dependent metalloproteases. Similarly to ubiquitination, deubiquitination is a highly regulated cellular process involved in diverse functions including cell cycle regulation (Song and Rape, 2008), proteasome- and lysosome-dependent protein degradation (Guterman and Glickman, 2004; Komada, 2008), and DNA repair (Kennedy and D'Andrea, 2005), among others.

The ubiquitin-proteasome system and the autophagy-lysosome system in protein quality control

Two major pathways of protein degradation have been described for most eukaryotic cellular proteins, the ubiquitin-proteasome system (UPS), and autophagy. The

UPS is responsible for degrading an estimated 80-90% of proteins including regulated, short-lived, or damaged proteins, while autophagy is primarily responsible for degrading long-lived proteins, aggregated proteins, and cellular organelles (Lilienbaum, 2013). Both of these degradation pathways are essential for maintenance of cellular homeostasis and are components of cellular protein quality control (PQC), a system that identifies, tags, and degrades misfolded or damaged proteins.

Ubiquitin-proteasome system (UPS)

Ubiquitin-dependent degradation by proteasomal components is the predominant mode of protein degradation and regulates many cellular processes including PQC, DNA repair, apoptosis, and receptor-mediated endocytosis (Bingol and Schuman, 2004; Lilienbaum, 2013; Ramadan and Meerang, 2011; Sarhan et al., 2014). Substrates for this degradation pathway are first tagged with ubiquitin. K48-linked polyubiquitination is the canonical signal for degradation via the UPS. A chain of four or more ubiquitins is generally the minimum requirement for substrate recognition by the 26 S proteasome (Thrower et al., 2000), though monoubiquitin targeting to the proteasome has also been described (Kravtsova-Ivantsiv and Ciechanover, 2012).

The 26 S proteasome is made of two subcomplexes, the core 20 S proteasome and the 19 S regulatory particle. The 20 S core is a barrel-shaped structure with a 2 nm central cavity diameter, made of two inner β-rings (made of seven β subunits) and two outer α rings (made of seven α subunits). The β subunits each have caspase-like, trypsin-like, or chymotrypsin-like activity that hydrolyze peptide bonds. The 19 S regulatory

particle is made of about 20 different proteins, forming a "lid" and a "base". The 19 S regulatory particle binds the 20 S core particle to open the core channel, as well as recognizes, unfolds, and translocates ubiquitinated proteins into the 20 S core in an ATP-dependent manner (Lilienbaum, 2013).

Because ATP is necessary for 19 S assembly with the 20 S core and for substrate unfolding and translocation into the 20 S core, low ATP in stressed cells can trigger autophagy upregulation (discussed further below) or ATP-independent protein degradation by the proteasome. Other protein complexes such as PA200, PA28, and PI31 can interact with the α-rings on the 20 S core particle, forming ATP-independent proteasome isoforms, though the physiological functions of these alternative protein complexes are poorly understood (Ma et al., 1992; Ustrell et al., 2002; Zaiss et al., 2002). Additionally, several alternative mechanisms for degradation that do not rely on ubiquitin and/or ATP have been described, including proteins that contain a signal for degradation, termed "degron" sequence (Rosenberg-Hasson et al., 1989), or oxidation-induced unfolding of proteins that do not require ATP or ubiquitin to be degraded by the 20 S core particle (Grune et al., 1997).

Autophagy-lysosome system

Autophagy refers to "self-eating" of the cell, where cellular components are degraded by lysosomes. Lysosomes are single-membrane vesicles that have a low internal pH of 4.5-5 and contain hydrolytic enzymes to degrade many substrates, including entire organelles (De Duve and Wattiaux, 1966; Mrschtik and Ryan, 2015).

The degradation products in many cases are recycled in the cell, or used to generate energy. There are three major types of autophagy which are differentiated by their mode of cargo delivery to lysosomes: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy.

Microautophagy involves sequestration of regions of the cytosol by invaginations of the lysosomal membrane itself. The small internalized vesicles that form pinch off into the lysosomal lumen for rapid degradation (Marzella et al., 1981; Park and Cuervo, 2013). This type of nonselective vesicle-mediated degradation has been well characterized in yeast, but has not yet been identified in mammals. However, a microautophagy-like process, endosomal microautophagy, was recently described to occur in late endosomes (Sahu et al., 2011). In this process, cargo is trapped in vesicular invaginations formed by chaperones and endosomal sorting complexes required for transport (ESCRT) machinery (Sahu et al., 2011).

CMA is a more selective form of autophagy where cytosolic hsc70 (heat shock cognate protein of 70 kDa) and other chaperones identify cargo and deliver it to lysosomes. Cytosolic proteins with the pentapeptide targeting motif KFERQ or related amino acid sequences are recognized by the hsc70 complex then bind to LAMP-2A (lysosome-associated membrane protein type 2A), the CMA receptor at the lysosomal membrane (Chiang et al., 1989; Cuervo and Dice, 1996; Dice, 1990). After unfolding of the substrate proteins, they are translocated into the lysosomes for degradation by a luminal form of hsc70 (Cuervo, 2010; Park and Cuervo, 2013). Two PD-linked proteins, α-synuclein and leucine-rich repeat kinase 2 (LRRK2), have been reported to undergo CMA. Pathogenic accumulation of aggregated α-synuclein is a major feature of Lewy

bodies found in PD and aggregates found in other neurodegenerative diseases (Baba et al., 1998; Farrer et al., 2004; Lippa et al., 2005). CMA plays an important role in α-synuclein clearance during conditions of increased protein burden such as oxidative stress (Mak et al., 2010). Mutant α-synuclein expression can also in turn cause CMA impairment, leading to aberrant α-synuclein toxicity and a compensatory induction of macroautophagy (Xilouri et al., 2009). LRRK2 can be degraded by lysosomes in CMA, but several pathogenic LRRK2 mutants were found to impair CMA, which could contribute to PD toxicity by inhibiting degradation of both mutant LRRK2 and α-synuclein (Orenstein et al., 2013). CMA has also been proposed to act as a compensatory mechanism when macroautophagy dysfunction occurs in early HD, as LAMP-2A and lysosomal hsc70 are increased in HD mouse models (Koga et al., 2011), suggesting some degree of cross-talk among autophagic pathways.

Macroautophagy is a highly conserved bulk degradation pathway that involves several steps. Through the coordination of several autophagy-related (Atg) proteins, cytosolic cargo is sequestered into a double-membraned autophagosome vesicle by elongation of a phagopore membrane. Then, the autophagosome fuses directly with a lysosome and hydrolases from the lysosomal lumen degrade the isolated cargo (Mizushima et al., 2011). Macroautophagy starts by release of inhibitory complexes (such as mTOR, the mammalian target of rapamycin) in response to various stimuli such as nutrient starvation or cellular stress (He and Klionsky, 2009). Macroautophagy is first initiated by nucleation of the phagophore or isolation membrane, a process that is controlled by the Beclin1-Vps35-Atg14 complex (Funderburk et al., 2010; Lilienbaum, 2013). Sources of phagophore membranes are not well characterized, but it is

hypothesized that the endoplasmic reticulum, Golgi complex, endosomes, and/or mitochondria could play a role (Kang et al., 2011; Mizushima et al., 2011). The second step, membrane vesicle elongation, is driven by Atg9 and WIPI (WD-repeat protein interacting with phospholipids) proteins (Longatti and Tooze, 2009; Mauthe et al., 2011). Microtubule-associated protein 1 light chain 3 (LC3-I), the mammalian ortholog of Atg8, localizes to autophagosomal membranes by the conversion of LC3-I to LC3-II (Levine and Kroemer, 2008). LC3-I is diffusely distributed in the cytoplasm, but shows a punctate distribution upon cleavage by Atg4 and lipidation to phosphatidylethanolamine (PE) by Atg7, Atg3, and the Atg12-Atg5-Atg16 conjugation complex to be incorporated into the autophagosomal membrane (Fujita et al., 2008; Hanada et al., 2007; Nakatogawa, 2013). The membrane vesicle then closes on itself to form a double-membraned vesicle (Cebollero et al., 2012; Xie et al., 2008), termed autophagosome, that engulfs cargo such as aggregated proteins and organelles. Autophagosomes can then fuse with endosomes from the endocytic pathway, which forms an amphisome (Berg et al., 1998). This structure then fuses with lysosomes to form an autolysosome, in which the cargo is finally degraded by lysosomal hydrolases in low pH (Mizushima et al., 2011). Overexpression of wild-type α-synuclein was recently reported to impair macroautophagy at a very early stage of autophagosome formation, leading to accumulation of aggregate-prone proteins and contributing to PD due to copy number expansion (Winslow et al., 2010). This finding suggests a role for macroautophagy dysfunction in the pathology of neurodegenerative diseases such as PD.

Contrary to the widely-held view that macroautophagy is a nonselective degradation process, mounting evidence suggests that selectivity of cargo can indeed

occur. Adaptor proteins such as p62/SQSTM1 (sequestome 1) and NBR1 (neighbor of BRCA1), which bind both ubiquitin via their UBA domains and LC3 via their LC3-interacting regions (LIR) (Johansen and Lamark, 2011; Pankiv et al., 2007), assist in anchoring ubiquitinated cargo to the autophagosome's inner cavity. p62 could play a role in ubiquitin-dependent selective autophagy, as it interacts preferentially with K63-linked polyubiquitin (Seibenhener et al., 2004) and has been found to link mutant SOD1 to LC3 (Gal et al., 2009). The adaptor protein Nix/BNIP3L is exclusively involved with mitochondrial degradation (mitophagy) during erythroid maturation, recruiting autophagosomes to depolarized mitochondria (Zhang and Ney, 2009). Besides ubiquitinated proteins and mitochondria, other groups have found that autophagosomes can also selectively target ribosomes (ribophagy), peroxisomes (pexophagy), and intracellular bacteria (xenophagy) (Kim et al., 2008; Kraft et al., 2008; Nakagawa et al., 2004).

1.2 Protein misfolding in neurodegenerative disease

Insoluble protein aggregates are characteristic of age-related neurodegenerative disorders collectively referred to as “protein misfolding diseases” (Gregersen, 2006; Whatley et al., 2008), including PD and ALS. Proteins can become misfolded through genetic mutations or environmental insults such as oxidative damage (Martins and English, 2014). An estimated 30% of all nascent proteins are defective ribosomal products (Schubert et al., 2000) that need to be refolded or degraded, thus subjecting cells to a continuous flux of misfolded proteins that must be cleared from the cell. Misfolded

proteins are normally cleared by efficient protein quality control systems such as molecular chaperones and the UPS (Goldberg, 2003). However, when these systems become impaired or are overwhelmed, misfolded proteins accumulate to form toxic oligomers (Olzmann and Chin, 2008; Olzmann et al., 2008). Insoluble protein aggregates found in diseased tissue (summarized in Table 1.1) resemble aggresomes, a specialized type of cellular inclusion body (Kopito, 2000), and reflect a failure of the proteasome to clear potentially toxic proteins (Kabashi and Durham, 2006).

Aggresome-autophagy pathway

Aggresome formation is another cellular defense mechanism, sequestering toxic misfolded proteins via dynein-mediated retrograde transport to prevent their interference with normal cellular function and facilitating bulk disposal via macroautophagy (hereafter referred to as autophagy) (Iwata et al., 2005; Kopito, 2000; Olzmann et al., 2008). Aggresomes are formed by dynein-mediated microtubule trafficking of smaller aggregates towards the microtubule organizing center (Ravikumar et al., 2005). Mounting evidence suggests that aggresomes are indeed autophagy substrates. Autophagy is a lysosome-dependent bulk degradation process that can be induced by proteasomal dysfunction (Bedford et al., 2009) or nutrient deprivation (Young and La Spada, 2009), although it also occurs at a homeostatic level. Upon autophagy induction, damaged proteins and/or organelles are sequestered into an autophagosome, which fuses with lysosomes to degrade its contents. Autophagic machinery is localized to aggresomes (Iwata et al., 2005; Yamamoto et al., 2006), and inhibition of autophagy blocks aggresome clearance (Fortun et al., 2003; Ravikumar et al., 2002), suggesting that

aggresomes are recognized and cleared by autophagic machinery in a process called the aggresome-autophagy pathway. Autophagy does not require unfolding of substrate proteins and can degrade large protein aggregates and entire damaged organelles. Therefore, aggregate-prone proteins may depend more heavily on autophagy for clearance than proteasomal degradation, since aggregated species cannot access the narrow proteasome barrel and can actually impair UPS function (Bence et al., 2001; Bennett et al., 2005).

Aggresome formation is thought to be cytoprotective because toxic, aggregated proteins are sequestered away from other cellular machinery and may undergo autophagic degradation (Fortun et al., 2003; Iwata et al., 2005; Tanaka et al., 2004; Taylor et al., 2003). Aggresome-forming proteins are associated with accelerated turnover rate, become membrane-bound, may undergo lysosomal degradation, and decrease cellular toxicity (Tanaka et al., 2004; Taylor et al., 2003). Although to date, aggresomes are a phenomenon studied in cell culture and may not necessarily fully represent inclusions found in neurodegenerative disease tissue, studies of aggresome formation have provided key insights into the molecular mechanisms underlying cellular management of misfolded proteins (Olzmann et al., 2008). Despite a growing body of research on this only recently described cellular process, the molecular mechanisms involved in targeting of misfolded proteins to the aggresome-autophagy pathway are still unclear.

Parkinson disease

Parkinson disease is a chronic, progressive neurodegenerative disorder that affects at least 1% of the population by age 70 (Savitt et al., 2006). It is projected that by the year 2030, there will be about 9 million people affected (Dorsey et al., 2007), highlighting the need for better understanding of PD etiology and effective treatments as the disease burden increases with the aging population. The clinical motor characteristics of PD include resting tremor, rigidity, postural and gait impairment, and bradykinesia (Massano and Bhatia, 2012). Non-motor symptoms include mood disorders, dementia, sleep disorders, and autonomic dysfunction, all of which can severely affect the patient's quality of life even before motor symptom onset (Massano and Bhatia, 2012; Seppi et al., 2011). PD is characterized pathologically by neuronal loss, especially in the dopaminergic neurons of the substantia nigra pars compacta (SNpc), and the presence of Lewy bodies and Lewy neurites in multiple areas of the brain (Braak et al., 2003; Marsden, 1983).

The majority of research on PD pathogenesis has focused on dopaminergic cell loss and Lewy bodies seen in the SNpc (Savitt et al., 2006). This has led to treatments aimed at the link between nigrostriatal dopamine loss and motor symptoms, such as the metabolic precursor of dopamine levodopa in combination with a peripheral decarboxylase inhibitor. However, prolonged use of levodopa leads to further motor complications such as levodopa-induced dyskinesia (Al Dakheel et al., 2014; Aquino and Fox, 2015). Other promising drugs from clinical trials include istradefylline, an adenosine A2 antagonist (Hauser et al., 2003), and sarizotan, a serotonin agonist (Olanow et al., 2004a), however, levodopa continues to be the main symptomatic therapy for the

motor symptoms of PD (Al Dakheel et al., 2014; Savitt et al., 2006). Deep brain stimulation (DBS), electrical stimulation of the globus pallidum internus (GPi) or subthalamic nucleus (STN), has also proven to be effective in improving motor symptoms, but this invasive procedure is generally reserved for advanced PD cases with severe motor complications and/or medication intolerance (Piper et al., 2005). An increased understanding of PD pathogenesis will help drive the development of more effective therapeutics.

PD is considered to be a multi-faceted disease caused by both genetic and environmental factors (Gao et al., 2011). The majority (~90-95%) of PD cases are sporadic, although aging is the most prominent disease risk factor (Rodriguez et al., 2015). Other risk factors include neuroinflammation and oxidative stress, environmental toxicant exposure, and the male sex (Burns et al., 1983; Smith and Dahodwala, 2014; Taylor et al., 2013). The remaining ~5-10% of PD cases are linked to genetic abnormalities in several genes, some highlighted below.

A mutation in the gene encoding α-synuclein (PARK1 and PARK4) was the first genetic mutation identified to cause PD (Polymeropoulos et al., 1997). PARK1-linked PD is caused by missense mutations in α-synuclein (Polymeropoulos et al., 1997) and PARK4-linked by multiplications of α-synuclein (Singleton et al., 2003). α-synuclein is an abundant presynaptic phosphoprotein that is a major component of Lewy bodies. Abnormal aggregation of overexpressed wild-type and mutated α-synuclein into toxic, misfolded oligomers could contribute to neuronal cell death (Moore et al., 2005). Lewy bodies, intracellular fibrillar aggregates that are a histopathological hallmark of PD as well as AD and the related dementia with Lewy bodies (DLB), are reported to contain

many different PD-linked proteins including α-synuclein, LRRK2 (leucine-rich repeat kinase 2), parkin, and PINK-1 (Kotzbauer et al., 2001; Wakabayashi et al., 2013). Whether Lewy bodies are beneficial or noxious is unknown; however, there is more substantial evidence for their protective effect. When the UPS is overwhelmed, Lewy bodies may isolate potentially toxic aberrant proteins and provide protection for the cell if they can be disposed of via autophagy or pushed into the extracellular space (Harrower et al., 2005; Lu et al., 2005; Olanow et al., 2004b; Tanaka et al., 2004). Alternatively, Lewy bodies themselves may accumulate and contribute to toxicity due to sequestration of other essential proteins (Harrower et al., 2005; Lu et al., 2005). Mounting evidence suggests that Lewy bodies may represent a cytoprotective mechanism in PD. Proteasome inhibition causes α-synuclein inclusion formation, but blocks dopaminergic cell death in cultured rat primary neurons (Sawada et al., 2004). In a *Drosophila* model of PD, an increased number of α-synuclein aggregates is correlated with reduced toxicity of α-synuclein (Chen and Feany, 2005). In addition, Lewy bodies have been shown to contain many proteins related to mitochondria, UPS, autophagy, and aggresomes (reviewed in Wakabayashi et al., 2013), indicating cellular attempts to contain and dispose of damaged proteins.

Disrupted ubiquitin signaling in PD

Age-related decreases in proteasome activity and the accumulation of ubiquitin-conjugated proteins illustrate the importance of functional ubiquitin signaling in normal health. Decreased proteasome activity weakens the cellular capacity to rid damaged proteins, and ubiquitinated proteins that are tagged but not efficiently removed may

contribute to toxicity (Lilienbaum, 2013; Low, 2011). Genetic studies have identified mutations in proteins involved in the ubiquitin signaling pathway such as parkin (Kitada et al., 1998) and ubiquitin C-terminal hydrolase L1 (UCH-L1) (Wintermeyer et al., 2000) in familial PD, which suggests that disrupted ubiquitin signaling can contribute to PD.

Mutations in parkin account for the majority of autosomal recessive PD cases (Lee and Liu, 2008). A homozygous deletion of exons 3-7 of the parkin (PARK2) gene was first described in autosomal recessive juvenile PD (Kitada et al., 1998). Parkin is an E3 ubiquitin-protein ligase that plays a role in a concerted chain of events resulting in ubiquitination of substrate proteins (Fang and Weissman, 2004; Hershko et al., 1983; Weissman, 2001). Parkin is capable of facilitating monoubiquitination, K48-linked polyubiquitination, and K63-linked polyubiquitination, highlighting diverse roles of parkin in the ubiquitination of different substrates and cellular pathways (Doss-Pepe et al., 2005; Joch et al., 2007; Lim et al., 2005). As such, parkin ubiquitination has been linked to UPS-mediated protein turnover (Rankin et al., 2011), mitophagy (Narendra et al., 2008), NF-kB signaling (Sha et al., 2010), and receptor trafficking (Fallon et al., 2006).

We previously showed that parkin selectively targets the PD-linked L166P DJ-1 mutant protein to the aggresome-autophagy pathway by K63-linked polyubiquitination (Olzmann and Chin, 2008; Olzmann et al., 2007). The K63-linked polyubiquitin is recognized by the dynein adaptor protein histone deacetylase 6 (HDAC6) and recruited to perinuclear aggresomes via retrograde transport (Chin et al., 2010; Kawaguchi et al., 2003). Loss-of-function mutations in parkin thus contribute to defective degradation pathways and cause early-onset PD almost entirely devoid of Lewy bodies (Farrer et al.,

2001; Mori et al., 1998; Takahashi et al., 1994), the pathological hallmark of PD-specific protein aggregation. Parkin is cytoprotective against accumulated β-amyloid implicated in AD (Rosen et al., 2010) as well as mutant huntingtin implicated in HD (Rubio et al., 2009). It has been shown that overexpression of parkin in the presence of proteasome inhibitors such as MG132 leads to aggresome-like perinuclear inclusions that are immunoreactive for parkin and α-synuclein (Junn et al., 2002). Together, these data show a role for parkin in aggresome formation and cellular health.

UCH-L1 is a predominantly neuronal deubiquitinating enzyme that is important for the generation of monomeric ubiquitin (Osaka et al., 2003; Wilkinson et al., 1989). A missense mutation in UCH-L1 (I93M) was found in a family with autosomal dominant PD (Leroy et al., 1998). UCH-L1 I93M was reported to have about 50% reduction in catalytic activity, which could lead to reduced turnover of UCH-L1's (unknown) substrates and increased aggregation of those substrates (Leroy et al., 1998). Our lab has previously shown that soluble UCH-L1 protein levels are reduced in the cortex from sporadic PD and AD patient brains (Choi et al., 2004a), and others have found a similar trend in patients with DLB (Barrachina et al., 2006). In addition, UCH-L1 was found in Lewy bodies and neurofibrillary tangles (Choi et al., 2004a; Lowe et al., 1990), indicating that a disruption of normal DUB activity levels and turnover may contribute to these diseases. UCH-L1 has also been implicated in childhood-onset neurodegeneration with optic atrophy (Bilguvar et al., 2013), ALS (Poon et al., 2005), and cancer (Brinkmann et al., 2013), suggesting a role for disrupted ubiquitin signaling in several distinct diseases.

Amyotrophic lateral sclerosis

ALS is the most common adult-onset motor neuron disease, characterized by selective degeneration of upper motor neurons (cortical neurons that innervate lower motor neurons) and lower motor neurons (neurons that directly innervate skeletal muscle) (Andersen, 2006). This degeneration produces progressive muscle atrophy and weakness beginning in limb or bulbar muscles, and eventually spreads to respiratory muscles leading to respiratory failure, usually within three to five years of onset (Logroscino et al., 2008; Rothstein, 2009; Wijesekera and Leigh, 2009). Non-motor symptoms of ALS can include cognitive impairment, depression, and anxiety (Gordon, 2013). There is currently no cure for ALS, so clinical care currently focuses on maintaining quality of life, especially for respiratory support. Riluzole is the only drug available for treating ALS, but only prolongs life on the scale of a few months (Miller et al., 2012). Supportive care used in conjunction with Riluzole, especially in advanced ALS, can include nutritional supplements/gastrostomy, ventilatory support, and the use of anxiolytics and opioids to relieve non-motor symptoms (Gordon, 2013).

An estimated 90-95% of ALS cases are sporadic (sALS), and the remaining 5-10% of cases are familial (fALS) (Rothstein, 2009) (Genetic loci associated with ALS are shown in Table 1.2). Hypotheses for the etiology of sALS include exposure to environmental toxicants or heavy metals, intense physical activity, physical trauma, cigarette smoking, and electrical shocks, among other potential risk factors (Gawel et al., 1983; Johnson and Atchison, 2009; Qureshi et al., 2006; Trojsi et al., 2013).

About 20% of fALS cases are attributed to mutations in the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) (Andersen, 2006; Rosen et al., 1993). Over 170

ALS-related mutations within SOD1 have been reported, most resulting in a toxic gain-of-function rather than enzymatic deficiency (Abel et al., 2012) (see ALSoD, ALS online genetics database, http://alsod.iop.kcl.ac.uk/). There is currently no evidence correlating enzyme activity levels to disease severity. SOD1 mutations in ALS do not necessarily decrease enzymatic activity, as mice expressing human SOD1 mutations develop ALS-like symptoms despite higher SOD1 activity than wild type mice (Gurney et al., 1994).

Evidence for dysfunction of intracellular protein degradation systems and subsequent contribution to disease pathogenesis also comes from other ALS-linked proteins. ESCRT complexes are crucial for sorting integral membrane proteins into multivesicular bodies and their lysosomal degradation (Filimonenko et al., 2007). Mutations in charged multivesicular body protein-2B (CHMP2B), an ESCRT-III subunit, are associated with ALS (Cox et al., 2010). Dysfunctional autophagy pathways are also likely in cases of ALS associated with CHMP2B mutations, as cells expressing these mutations have inhibited autophagy and accumulated ubiquitin- and p62-positive aggregates (Filimonenko et al., 2007).

Ubiquilin 2 is a ubiquitin-like protein that regulates degradation of ubiquitinated misfolded proteins (Mah et al., 2000). Mutations in ubiquilin 2 result in impaired proteasomal degradation in ALS, which leads to protein aggregation and neurodegeneration (Deng et al., 2011). These studies highlight the contribution of defective ubiquitin-mediated protein degradation pathways to ALS.

Cellular mechanisms that may contribute to ALS -- Since many apparently unrelated genes have been linked to ALS, it is hypothesized that ALS could represent a common final endpoint for different physiological systems. There are several molecular

pathways that may be involved in ALS pathogenesis. One hypothesis for ALS pathogenesis is excitotoxicity triggered by overstimulation of glutamate receptors. The correlation of increased glutamate levels in the cerebrospinal fluid of ALS patients with disease severity supports this view (Rothstein et al., 1990; Spreux-Varoquaux et al., 2002). In addition, end-stage rodent transgenic ALS models have reduced levels of EAAT2 (astrocytic glutamate transporter; responsible for clearing synaptic glutamate) in spinal cord (Bruijn et al., 1997; Howland et al., 2002). Riluzole, the only FDA-approved drug available in delaying ALS fatality to date, has anti-glutamatergic activity, though its exact mechanism of action is uncertain (Gibson and Bromberg, 2012; Lacomblez et al., 1996).

An imbalance between reactive oxygen species generation and removal or repair of damage can lead to cytotoxic oxidative stress (Bogdanov et al., 2000). Mutations in SOD1, which is a major antioxidant protein, initiated an interest in the role of oxidative stress in ALS (Simpson et al., 2003). In studies of cerebrospinal fluid and blood samples from ALS patients, several markers of free radical damage have been identified (Turner et al., 2009). However, altered catalytic activity of mutant SOD1 may not represent the entire cause of oxidative stress-induced cytotoxicity, since enzymatically inactive SOD1 can still cause motor neuron degeneration (Wang et al., 2003).

Mitochondrial dysfunction has also been implicated in ALS. Even though SOD1 is predominantly cytosolic, a subset of SOD1 protein localizes to mitochondria, and mutant forms of SOD1 accumulate on the outer mitochondrial membrane and inside the mitochondrial matrix (Kawamata and Manfredi, 2008; Vijayvergiya et al., 2005). Misfolded mutant SOD1 is reported to inhibit components of mitochondrial signaling and

transport such as the voltage-dependent anion channel (VDAC1) and translocase of the outer membrane (TOM) complex (Israelson et al., 2010; Li et al., 2010). Mutant SOD1-induced mitochondrial damage then causes altered mitochondrial morphology and distribution, which correlates with disease severity (Vande Velde et al., 2011).

Neuroinflammation is also proposed to contribute to ALS pathogenesis. Cerebrospinal fluid and blood samples from ALS patients have activated macrophages and elevated levels of inflammation markers such as chemokines and interleukins (Kuhle et al., 2009; Mitchell et al., 2009; Zhang et al., 2005; Zhang et al., 2009a). ALS rat models have demonstrated microglia and macrophage activation in the spinal cord (Graber et al., 2010), and these changes have been linked to ALS disease progression (Zhang et al., 2009a). Further studies are needed to determine the relative contributions of these proposed pathogenic pathways to ALS pathology and guide future therapeutic development.

Mutations in two RNA binding proteins, FUS and TDP-43, suggest that errors in RNA metabolism could contribute towards ALS pathogenesis. TDP-43 (transactive response DNA binding protein 43 kDa) is a nuclear protein encoded by the *TARDBP* gene that regulates transcription and mRNA splicing, and mutations in TDP-43 are reported in both fALS and sALS (Neumann et al., 2006). Degradation of TDP-43 by both the proteasome and autophagy has been reported (Wang et al., 2010). TDP-43 is a major component of protein aggregates found in ALS, due to aberrant cleavage of TDP-43 to generate fragments that lack the nuclear localization signal and instead aggregate in the cytoplasm (Zhang et al., 2009b). Mutations in another RNA binding protein, FUS (encoded by the *fused in sarcoma/translocated in liposarcoma* or *FUS/TLS* gene), were

found to account for about 5% of fALS cases (Kwiatkowski et al., 2009; Vance et al., 2009). FUS inclusions (also containing TDP-43 and ubiquitin) are reported in both sALS and fALS, excluding cases caused by mutations in SOD1 (Deng et al., 2010). It is possible that both TDP-43 and FUS, which have been shown to interact (Ling et al., 2010), could function together in a common pathway that is defective in ALS (Deng et al., 2010; Freibaum et al., 2010; Kim et al., 2010).

Ubiquitin dysregulation in ALS

Pathologically, ALS is characterized by protein aggregates including Lewy bodies, skein inclusions, and Bunina bodies (Pasinelli and Brown, 2006). SOD1 has been found in both sALS and fALS inclusion bodies (Andersen, 2006; Cereda et al., 2013), suggesting a common degradation pathway failure and subsequent accumulation of both damaged wild-type SOD1 and mutant SOD1. Mutant SOD1 is reported to undergo both proteasomal and autophagic degradation (Kabuta et al., 2006). However, misfolded, aggregated SOD1 found in ALS tissues reflect a failure of molecular chaperones and UPS components to efficiently degrade mutant SOD1 (Kabashi and Durham, 2006). Recent evidence supports the possibility that defective autophagic pathways may cause toxic accumulation of SOD1 and contribute to ALS etiology. The autophagy enhancer rapamycin decreased SOD1 aggregates in cells and also reduced mutant SOD1-induced toxicity (Kabuta et al., 2006). Mutant SOD1 can be recognized by ubiquitin-autophagic membrane adapter protein p62 to promote autophagic degradation of mutant SOD1 (Gal et al., 2009). Together, these data suggest a role for autophagy in clearance of aberrant SOD1 proteins. On the other hand, mutant SOD1 was also reported to inhibit autophagic

machinery and lead to defective clearance (Zhang et al., 2007). Dynein is an essential component of autophagosome trafficking to lysosomes (Kimura et al., 2008; Webb et al., 2004). Mutant SOD1 was shown to interact with dynein and impair axonal transport, leading to accumulation of mutant SOD1 and dynein in mouse spinal cord neurons (Zhang et al., 2007). This raises the possibility that mutant SOD1 itself may also contribute to autophagic dysfunction in ALS.

The SOD1 A4V mutation is the most frequent mutation in North America, and also one of the most lethal (Gaudette et al., 2000). Although rare in humans, the G93A mutation has been extensively studied in animal models (Cheroni et al., 2009; Cheroni et al., 2005; Kieran et al., 2005; Nagai et al., 2001). In its native form, SOD1 is expressed as a stable homodimer, but ALS-associated mutations impair dimerization and lead to pathological monomers and subsequent aggregation (Keerthana and Kolandaivel, 2015; Rakhit et al., 2004; Rakhit et al., 2007). Mutations in SOD1, including A4V and G93A, destabilize the SOD1 dimer and cause misfolded conformations (Galaleldeen et al., 2009; Schmidlin et al., 2009) that form cytotoxic oligomers (Furukawa and O'Halloran, 2005; Rakhit et al., 2004). A large portion of SOD1 A4V adopts a misfolded conformation (identified by epitope-specific antibodies (Prudencio and Borchelt, 2011)), furthering the possibility that misfolded SOD1 forms oligomers and larger aggregates after protein misfolding occurs. Recent evidence shows that misfolded SOD1 aggregation can also cause aggregation of native SOD1, similar to the infectious prion protein (Polymenidou and Cleveland, 2011).

The pathology of sALS, like that of fALS, includes SOD1-containing inclusions (Fischer et al., 2004; Gerstner et al., 2008), as well as increased levels of oxidatively

damaged proteins (Ferrante et al., 1997; Shaw et al., 1995). Misfolded SOD1 is a common link between fALS and sALS (Perez-Garcia and Burden, 2012b). In sALS, WT SOD1 can become misfolded by oxidative damage which causes it to become structurally unstable, aggregating and forming inclusions (Fischer et al., 2004; Gerstner et al., 2008; Hough et al., 2004; Rakhit et al., 2004) similarly to mutant SOD1, due to increased binding and toxic properties (Sasaki, 2011). sALS patients also have increased levels of oxidatively damaged proteins (Parone et al., 2013; Song et al., 2012) and failure in protein quality control mechanisms (Vijayvergiya et al., 2005). These inclusions are likely due to the increased binding and toxic properties of oxidized WT SOD1 (Ezzi et al., 2007), which cause it to aggregate (Rakhit et al., 2004) similarly to mutant SOD1. In addition, our lab has identified oxidatively modified SOD1-containing aggregates in AD and PD brains (Choi et al., 2005). Together, these findings suggest that misfolded SOD1 may play a role in the pathophysiology of various neurodegenerative diseases, including ALS.

Proteasome function was reportedly altered in *SOD1*^{G93A} transgenic mice, leading to accumulation of SOD1- and ubiquitin-containing inclusions and UPS impairment in motor neurons specifically (Cheroni et al., 2009; Cheroni et al., 2005). Mutant SOD1 can be degraded by the UPS via polyubiquitination by the E3 ligase, Dorfin, which was found to prevent SOD1-mediated neurotoxicity (Niwa et al., 2002). However, the specific type of polyubiquitin linkage was not identified in this study. In addition, mutant but not WT SOD1 was polyubiquitinated under proteasomal inhibition with MG132, and overexpression of Dorfin reduced the number of mutant SOD1-containing aggregates and

protected against mutant SOD1 toxicity, but the authors did not explore the possibility of autophagic degradation under conditions of proteasome impairment.

The A4V mutant SOD1 was recently reported as a substrate for K63-linked polyubiquitination under proteasomal inhibition, a signal associated with facilitating selective clearance of inclusions via autophagy (Tan et al., 2008), though the E3 ligase responsible for its polyubiquitination has not yet been identified. In addition, these misfolded SOD1-containing inclusions are located in the perinuclear area, showing similar location and morphology to aggresomes. Further evidence to support SOD1 degradation via autophagy includes the finding that SOD1 mutants associate with the ubiquitin-binding protein p62, a common component of cytoplasmic inclusions found in protein aggregation diseases including ALS (Mizuno et al., 2006), which in turn interacts with LC3, a protein essential for activation of autophagy (Gal et al., 2009). p62 was also found to enhance formation of SOD1 mutant aggregates (Gal et al., 2007). These results suggest that p62 functions as an ‘adaptor’ between ubiquitinated SOD1 mutants and autophagic machinery. It was recently reported that HDAC6, an adaptor protein that links K63-linked polyubiquitin chains and the dynein motor complex (Olzmann et al., 2007), selectively interacts with the A4V mutant form of SOD1 (Gal et al., 2013) via two SMIR motifs previously identified in p62. Here, HDAC6 knockdown increased aggregation of mutant SOD1; however, the contribution of MG132-induced proteasome inhibition was not assessed in this study.

Inclusions isolated from both ALS mouse models and fALS patients are immunopositive for mutant SOD1 and ubiquitin (Polymenidou and Cleveland, 2011). In addition, cellular studies show that mutant SOD1 misfolds to form insoluble, ubiquitin-

positive, toxic protein aggregates which must be cleared from cells (Basso et al., 2006; Imai and Lu, 2011; Johnston et al., 2000; Niwa et al., 2002; Yonashiro et al., 2009). Others have shown that mutant SOD1 can be degraded by cellular quality control systems requiring ubiquitin signaling, including both proteasomal and autophagic degradation (Kabuta et al., 2006). The E6-AP E3 ubiquitin protein ligase was recently shown to ubiquitinate mutant SOD1 and facilitate its clearance (Mishra et al., 2013), though the ubiquitin linkage and degradation pathway were not identified. Despite these important findings, the ubiquitination pattern(s) of mutant SOD1 have not been well-characterized, and the molecular machinery required for SOD1 ubiquitination and subsequent clearance remain largely unknown.

1.3 Mouse models of neurodegenerative disease

Both PD and ALS are neurodegenerative diseases that are mostly sporadic, with only a fraction of cases caused by known genetic mutations. However, in both diseases, the pathology of familial forms is often indistinguishable from sporadic forms. Therefore, studying known mutations, particularly in animal models, can provide valuable insight into common molecular pathways in both familial and sporadic disease. Animal models that recapitulate the pathological hallmarks and characteristic neurodegeneration found in neurodegenerative diseases such as PD and ALS are important tools for testing therapeutics and validating molecular pathways *in vivo*. Mouse models are preferred in the context of studying human genetic disorders because of the similarities between mice and humans in neuronal networks and gene homologs

(Rosenthal and Brown, 2007; Waterston et al., 2002). In addition, the long lifespan compared to other, simpler model organisms allows researchers the benefit of studying disease pathology in aging, which is the greatest risk factor for many neurodegenerative diseases (Lee et al., 2012c).

PD mouse models

Neurotoxin-based models -- Many early PD models used the neurotoxin 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), which produces neurodegeneration of dopaminergic neurons, akinesia, rigidity, tremor, and gait/posture abnormalities similarly to the idiopathic disease (Arai et al., 1990; Langston et al., 1983; Langston et al., 1999). In addition, the behavioral symptoms can be reversed with dopamine substitution, which is the main therapeutic approach for PD (Kaakkola and Teravainen, 1990). MPTP is a protoxin that is converted to 1-methyl-4-phenylpyridinium (MPP^+), the toxic metabolite, in the brain (Heikkila et al., 1984). MPP^+ has a high affinity for the dopamine transporter, and thus enters dopaminergic neurons easily. MPP^+ impairs mitochondrial respiration by inhibiting complex I of the mitochondrial electron chain, resulting in increased ROS production and cell death (Nicklas et al., 1985). The MPTP mouse model has been classically used to study molecular pathways involved in PD neuronal death.

Another widely-used neurotoxin, 6-hydroxydopamine (6-OHDA), possesses a chemical similarity to catecholamines and thus has a high affinity for catecholamine transporters and can damage catacholaminergic neurons (Luthman et al., 1989). The

toxicity of 6-OHDA is due to the oxidative stress triggered by ROS production after entering neuronal transporters (Cohen, 1984). Injected into the SNpc, 6-OHDA can cause neurodegeneration of dopaminergic neurons within 24 hours (Faull and Laverty, 1969). This type of treatment generates a partial lesion that resembles PD dopaminergic cell loss. The 6-OHDA striatal unilateral lesion model is commonly used for studying a rotational or circling behavior in rodents. Compensatory mechanisms, such as the increase of dopamine D2 post-synaptic receptor density, are activated to make up for the decrease of dopamine. Rotational behavior is due to asymmetry in dopaminergic neurotransmission between the lesioned and intact striata, and rodents rotate away from the side of greater dopaminergic activity. Dopaminergic agonist administration induces a contralateral rotation (away from lesioned side) due to direct action on the hypersensitized dopamine receptors on the lesioned side, but drugs that stimulate dopamine release on the presynaptic side result in ipsilateral rotation (toward the lesioned side) by blocking dopamine reuptake and increasing dopamine receptor activity on the intact side (Bove and Perier, 2012). Since the rate of rotation correlates with the lesion's severity, rotation has been used to determine efficacy for new pharmacological agents to bind dopamine receptors and to establish a model for underlying motor complications in levodopa-induced dyskinesia (Bove and Perier, 2012).

Exposure to the pesticide rotenone has been linked to a higher risk of PD (Dhillon et al., 2008). Rotenone inhibits mitochondrial complex I to impair oxidative phosphorylation, and also inhibits microtubule formation (Marshall and Himes, 1978; Schuler and Casida, 2001). Chronic rotenone treatment of rats induces inclusion formation, something that was not found in 6-OHDA and MPTP models (Betarbet et al.,

2000). In mice, rotenone causes selective nigrostriatal degeneration (Inden et al., 2011). The herbicide paraquat is another neurotoxin that induces oxidative stress (Przedborski and Ischiropoulos, 2005) and has been linked to increased PD risk (Wang et al., 2011). Mice treated with paraquat have shown reduced motor activity and loss of striatal dopaminergic nerve fibers and substantia nigra neuronal cell bodies (Brooks et al., 1999); however, there is some debate as to the reproducibility of paraquat-induced degeneration among the age and strain of mice treated (Bove and Perier, 2012).

Transgenic rodent models -- Transgenic animals that overexpress a foreign protein (such as disease-linked wild-type or mutant genes) have been developed to model several human diseases in mice (Rockenstein et al., 2007). Because aggregation of α-synuclein plays a major role in the pathogenesis of PD (Moore et al., 2005), most transgenic mouse models of PD have centered around α-synuclein accumulation (Fernagut and Chesselet, 2004; Hashimoto et al., 2003). Overexpression of WT α-synuclein under the regulatory control of the neuron-specific PDGF-β (platelet-derived growth factor-β) promoter results in motor deficits when evaluated on the rotarod, striatal dopaminergic terminal loss, and ubiquitin- and α-synuclein-positive inclusion body formation (Masliah et al., 2000). No neuronal loss was detected in the substantia nigra, but a reduction of 25-50% of dopamine levels in the caudoputamen region was found at 12 months of age. The α-synuclein A53T transgenic mouse (under mouse prion promoter, mPrP) reproduces phosphorylation, ubiquitination, and aggregation of α-synuclein seen in PD, but most of the neurodegeneration was found in motor neurons instead of dopaminergic neurons (Giasson et al., 2002). Other transgenic lines using neuronal promoters to express α-synuclein (WT or mutants) have been generated, but the severity

and age of disease onset depends on the promoter and the level of transgene expression (Giasson et al., 2002; Neumann et al., 2002; Nuber et al., 2008; Richfield et al., 2002). Though some models show region-specific neuron loss, none recapitulate dopaminergic cell loss, a central pathological feature of PD. Because limited dopaminergic neuron loss was reported in most α-synuclein mouse models, mechanistic studies on α-synuclein-related neuronal death have also used MPTP to target dopaminergic neurons. In MPTP mouse models, mitochondrial dysfunction can induce α-synuclein aggregation and dopaminergic neuron loss (Song et al., 2004). Additionally, α-synuclein transgenic mice have mitochondrial abnormalities in neurons undergoing degeneration (Martin et al., 2006) and higher sensitivity to mitochondrial toxins (Nieto et al., 2006), suggesting an interplay between α-synuclein and mitochondrial damage in the pathogenesis of PD.

Autosomal dominant mutations in LRRK2 are the most common cause of familial PD (Goldwurm et al., 2005). Abnormal LRRK2 kinase activity due to mutations is linked to dopaminergic neuron degeneration in PD (West et al., 2007), likely due to aberrant phosphorylation of substrates and misregulation of protein binding partners (Lee et al., 2012c). Several transgenic techniques for generating LRRK2-induced PD mouse models have been attempted (such as conventional transgenic, mutant LRRK2 knockin, and tet-inducible transgenic) (Dawson et al., 2010; Li et al., 2009; Wang et al., 2008), but the only LRRK2 model that recapitulates age-dependent nigral dopaminergic neuron death was described by Ramonet and colleagues (2011). In this model, overexpression of the pathogenic G2019S mutant LRRK2 resulted in high levels of transgene expression and specific neuronal loss in the substantia nigra (Ramonet et al., 2011).

Knockout models -- Since loss of function mutations in several PD-associated genes are linked to autosomal recessive PD (Kitada et al., 1998), targeted deletion of gene exons or premature termination of the gene rendering loss of function should be able to model human PD in mice. However, knockout (KO) of parkin, PINK1, or DJ-1, or even all three genes (Kitada et al., 2009) in mice have resulted in minimal PD phenotypes (Dawson et al., 2010).

Several groups have generated parkin KO mice as a model of PD caused by parkin loss (Goldberg et al., 2003; Itier et al., 2003; Perez and Palmiter, 2005; von Coelln et al., 2004). These initial parkin KO models did not produce dopaminergic neuron death in the substantia nigra, though abnormal dopamine metabolism and accumulation of parkin substrates was observed (Itier et al., 2003; Ko et al., 2005; Shin et al., 2011). *Parkin* exon 3 deletion mice had no behavioral modifications compared to control mice except reduced body weight and reduced exploratory behavior (Itier et al., 2003). Increased dopamine levels in limbic regions and increased dopamine metabolism to dihydroxyphenylacetic acid (DOPAC) and H_2O_2 were observed in these mice, as well as a possibly neuroprotective compensatory increase in the antioxidant reduced glutathione (Itier et al., 2003). A targeted deletion of *parkin* exon 2 resulted in normal catecholamine levels, no significant behavioral differences, and no dopaminergic neuron loss (Perez and Palmiter, 2005). The inconsistency among parkin KO mouse phenotypes regarding neuronal loss or behavioral differences could be attributed to background strain differences, redundant E3 ligases found in mice but not humans, yet unknown compensatory mechanisms, differences in the functions of mouse and human parkin, and/or the absence of environmental triggers (Perez and Palmiter, 2005). The *parkin*

exon 2 deletion mice described by Perez and Palmiter (2005) were not more sensitive to methamphetamine or 6-OHDA compared to control mice, though the authors hypothesize that other neurotoxins such as rotenone or MPTP may trigger parkinsonian phenotype in these mice via a different mechanism of action. However, MPTP treatment in exon 7 deletion parkin KO mice did not show increased vulnerability to MPTP toxicity compared to WT mice (Thomas et al., 2007). Though further studies will be needed to determine why parkin-null mice do not exhibit parkinsonian phenotypes, these mice still serve as a useful model for exploring parkin loss of function *in vivo*.

Loss of PINK1 kinase activity due to mutations are linked to early-onset PD in humans. PINK1-targeted KO mice and shRNA knockdown models have been reported, but these models do not show robust neurodegeneration or mitochondrial defects (Gispert et al., 2009; Kitada et al., 2007; Zhou et al., 2007). One PINK1 KO mouse model showed mild abnormalities in mitochondrial respiration and electrochemical potential (Gispert et al., 2009); however, since these models do not display dopaminergic neuron degeneration, PINK1 loss alone may not be the cause of PD pathology.

DJ-1 KO mice also develop mild mitochondrial dysfunction, but do not display nigral neurodegeneration (Chen et al., 2005; Goldberg et al., 2005). Consistent with the protective role DJ-1 plays against cellular redox stress (Moore et al., 2003), DJ-1 null dopamine neurons have increased susceptibility to MPTP toxicity (Kim et al., 2005). Altered mitochondrial function and oxidative stress (Guzman et al., 2010) despite lack of neurodegeneration in these mice implies that changes due to DJ-1 loss may predispose dopamine neurons for increased sensitivity to additional "hits" such as neurotoxin exposure and aging (Lee et al., 2012c).

The apparent lack of PD phenotype in the conventional KO approach has led to the use of conditional knockouts. The Cre-*loxP* conditional KO approach is often employed when studying a germline gene deletion that results in embryonic lethality (Nagy, 2000). Tissue-specific Cre-expressing mouse lines can be crossed with lines that have exons or genes flanked by *loxP* sites, which can be deleted in specific tissues when the lines are cross-bred. A variation on this method is to use a lentiviral vector expressing GFP fused to Cre recombinase, stereotaxically injected into desired brain regions (such as the substantia nigra of exon 7 floxed parkin mice) (Shin et al., 2011). In this model, conditional KO of parkin in adult mice resulted in progressive loss of dopamine neurons via dysregulation of Parkin Interacting Substrate (PARIS), a newly identified parkin substrate normally degraded by the UPS. This mouse model suggests a role for parkin loss in pathogenesis of PD. Although none of the currently reported PD mouse models recapitulates all of the features of PD in humans, mild deficits have been observed in some models, which may be useful for understanding some of the physiological changes that may precede neurodegeneration (Lee et al., 2012c).

ALS mouse models

Mutations in *SOD1* are found across all exons, and over 170 different mutations can induce the same disease phenotype in fALS (Abel et al., 2012). Therefore, SOD1 mutations were initially thought to cause a loss-of-function in the superoxide dismutase activity which is protective against oxidative stress (Joyce et al., 2011; Turner and Talbot, 2008). However, some ALS-linked mutations have normal superoxide dismutase activity, and some actually have increased activity (Borchelt et al., 1994) suggesting loss-

of-function is not a pathogenic mechanism in ALS. In addition, SOD1 KO mice do not display motor neuron degeneration (Reaume et al., 1996). Instead, SOD1 mutations are now thought to cause a toxic gain-of-function and several SOD1 mouse models have been created to study this hypothesis.

Mutant SOD1 transgenic mice -- The first animal model of ALS was the *SOD1*^{G93A} mouse, expressing human *SOD1* encoding the fALS-linked G93A mutation (Gurney et al., 1994). This mouse model remains the most widely used one in SOD1-related ALS research since it replicates many of the ALS phenotypes observed in humans such as progressive muscle atrophy and loss of motor neurons (Turner and Talbot, 2008). The commonly used *SOD1*^{G93A} high-expressing strain develops hindlimb tremor and weakness starting around 90 days (~3 months), which progresses to paralysis and endpoint by about 120 days (~4 months) (Gurney et al., 1994). Neuromuscular junction degeneration in fast-fatigable axons occurs around 47 days (~1.5 months) and about 50% of lower motor neurons are lost by 100 days (~3.3 months) (Fischer et al., 2004). Pathologically, these mice have mitochondrial vacuolization (Dal Canto and Gurney, 1995), SOD1-positive aggregates (Johnston et al., 2000), and astrocytosis and microgliosis of the spinal cord (Hall et al., 1998). Proteasome function is altered in *SOD1*^{G93A} transgenic mice, leading to accumulation of SOD1- and ubiquitin-containing inclusions and UPS impairment in motor neurons (Cheroni et al., 2009; Cheroni et al., 2005). In addition, inclusions isolated from both ALS mouse models and familial ALS patients are immunopositive for mutant SOD1 and ubiquitin (Polymenidou and Cleveland, 2011). Together, these data suggest involvement of the aggresome-autophagy pathway in cellular processing of misfolded SOD1.

ALS-like phenotypes vary among mutant SOD1 mouse models depending on the mutation and the transgene expression level. For example, *SOD1*^{G93A} mice that express high levels of transgene (25 predicted copies) develop the ALS phenotype earlier (onset ~90 days or ~3 months) (Chiu et al., 1995) than *SOD1*^{G93A} mice that have low transgene levels (8 predicted copies, onset ~170 days or ~5.7 months) (Gurney, 1997), suggesting a dose effect of mutant SOD1 (Joyce et al., 2011). The *SOD1*^{A4V} mouse does not develop ALS symptoms until late in life (>1.6 years), despite the A4V mutation causing a highly aggressive disease in humans (Siddique and Deng, 1996). SOD1 G37R is a stable and active enzyme with increased dismutase activity (Borchelt et al., 1994). *SOD1*^{G37R} mice develop hindlimb paralysis between 4 to 6 months of age and also extensive mitochondrial degeneration in the spinal cord (Wong et al., 1995). However, vacuolized mitochondria are rarely described in human ALS (Sasaki et al., 1998), which suggests this phenotype could be a result of exaggerated mutant SOD1 expression. *SOD1*^{G85R} mice also display ALS-like phenotypes (Bruijn et al., 1997) but this occurs late in life (after 8 months) and the disease has a very short progression (~2 weeks). In this model, SOD1 G85R has normal levels of enzymatic activity but SOD1 expression levels were below that of endogenous mouse SOD1. Transgenic expression of human SOD1 mutants in mice, from 0.2 to 25-fold protein expression and 0 to 14.5-fold SOD1 activity levels, have all resulted in fatal motor neuron degeneration in mice (Turner and Talbot, 2008) which is the primary evidence for a dominant gain-of-function in SOD1-linked ALS pathogenesis.

There are a couple of proposed contributing factors for the variation in disease phenotype seen in mice, besides the species difference with humans. A spontaneous

SOD1 E77K mutation in mice does not result in any phenotypical differences at one year of age (Luche et al., 1997). Although this mutation is not reported in ALS patients, this finding suggests that multiple copies of SOD1 transgenes may be a prerequisite for accelerated disease phenotype in mice, which have a shorter lifespan than humans (Turner and Talbot, 2008). In addition, mouse SOD1 lacks Cys-111 (Ferri et al., 2006), which has been proposed to modulate human SOD1 misfolding and aggregation-induced toxicity (Wang et al., 2006), requiring a destabilizing mutation in mouse SOD1 to elicit the disease phenotype in mice. Background strain differences have also been shown to affect disease onset and progression, as C56BL/6 (B6) *SOD1*^{G93A} mice survive longer than the fast-progressing B6/SJL *SOD1*^{G93A} mice (Mancuso et al., 2012).

Studies using treatment of *SOD1*^{G93A} mice with various drugs, injections of recombinant proteins, and cross-breeding strategies have provided some insight into underlying mechanisms of ALS, however, the positive findings have typically shown only mild increases in lifespan (see Table 1.3 for a comparison of various strategies in mice). Since Riluzole only increases lifespan by about 3 months in human ALS patients, much more research into therapeutic targets are needed for ALS treatment.

SOD1 knockout mice -- SOD1 KO mice were developed on the premise that SOD1 loss of function due to various mutations could all lead to ALS pathogenesis. However, SOD1 KO mice do not show motor abnormalities by 6 months of age or spinal cord pathology by 4 months of age (Ho et al., 1998; Reaume et al., 1996), but the mice are more vulnerable to motor neuron loss after axonal injury which suggests a role for SOD1 in injury, but not necessarily neuronal development (Reaume et al., 1996). Another SOD1 KO mouse study showed increased susceptibility to paraquat toxicity and

decreased fertility in female mice, linking free radicals to abnormalities in reproduction (Ho et al., 1998). SOD1 KO mice have more recently been shown to develop age-related (>1 year of age) peripheral axonopathy, denervation of hindlimb muscles, and accelerated loss of muscle mass (sarcopenia) (Flood et al., 1999; Muller et al., 2006), which suggest that loss of SOD1 may, in fact, contribute to some of the ALS disease phenotypes. Besides dismutase activity, SOD1 can also catalyze peroxidation, nitration, and thiol oxidation (Beckman et al., 1993; Winterbourn et al., 2002; Yim et al., 1990), among other functions, but these are not routinely tested in studies of SOD1 models, so the SOD1 loss-of-function hypothesis of these SOD1 functions cannot be fully dismissed (Turner and Talbot, 2008).

Wild-type SOD1 transgenic mice -- Mice overexpressing (high transgene copy number) human $SOD1^{WT}$ are often used as a control for the human $SOD1^{G93A}$ high-expressing mice since transgene copy number is similar but the $SOD1^{WT}$ overexpressing mice do not develop ALS motor phenotypes. However, $SOD1^{WT}$ overexpressing mice develop axonopathy, which suggests that SOD1 overexpression causes neuronal defects regardless of the presence of a pathogenic mutation (Jaarsma, 2006). $SOD1^{WT}$ transgenic mice are also reported to develop vacuolar pathology, axonal loss, and motor neuron degeneration in the spinal cords of mice at 30 weeks (7 months) old (Jaarsma et al., 2000). However, the lack of full ALS symptoms even in aged mice implies that these other phenotypes constitute subclinical motor neuron degeneration, termed "multi-system axonopathy" (Jaarsma, 2006). Therefore, wild-type SOD1 overexpression can cause some motor neuron degeneration. A hypothesized explanation for this contradictory finding, that SOD1 KO and $SOD1^{WT}$ overexpressing mice can both develop aspects of

disease phenotype, is that SOD1 expression has a biphasic effect where either extreme in SOD1 expression may be harmful (Xing et al., 2002). Therefore, intermediate levels of SOD1 expression may be the key for longer-term motor neuron health.

TDP-43 mouse models -- Several TDP-43 mouse models have been described since *TARDBP* mutations were linked to fALS (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Yokoseki et al., 2008). As with SOD1 mouse models, the observed phenotypes in these TDP-43 models vary with expression levels and the promoter used (Joyce et al., 2011). Overexpression of TDP-43 WT causes premature death in almost all models described, except the model using mouse prion promoter (highest expression levels in brain and heart) (Stallings et al., 2010). Wild-type TDP-43 overexpression has also been reported to cause varying degrees and onset of gait abnormalities, tremors, and paralysis (Shan et al., 2010; Wils et al., 2010; Xu et al., 2010).

A $TARDBP^{A315T}$ transgenic mouse was reported to develop progressive gait abnormalities starting ~13 weeks (~3 months) with premature death ~22 weeks (~5.1 months) (Wegorzewska et al., 2009). However, another group developed a $TARDBP^{A315T}$ transgenic mouse with similar protein expression levels and the same promoter that displayed a more aggressive disease phenotype, with premature death between 7.5 to 10.5 weeks (~1.7 to 2.5 months) (Stallings et al., 2010). Studies in TDP-43-null mice show that TDP-43 is essential for embryonic development, as homozygous $Tardbp^{-/-}$ embryos fail to mature past E7.5 (Kraemer et al., 2010; Sephton et al., 2010; Wu et al., 2010). $Tardbp^{+/-}$ mice show age-related muscle weakness, suggesting TDP-43 contributes

somewhat to motor neuron survival, but Kraemer et al. did not identify an associated ALS-like pathology.

FUS mouse models -- Two groups independently reported FUS KO mouse models before mutations in *FUS* were linked to ALS (Hicks et al., 2000; Kuroda et al., 2000). These studies showed that FUS is required for viability (newborn FUS KO mouse pups died within 16 hours of birth) and genome stability (FUS KO mice have chromosomal abnormalities such as aneuploidy, chromosomal fusion, and chromosomal breakage) (Hicks et al., 2000). Overexpressing FUS mutant R521C results in an ALS-like phenotype in rats, accumulation of ubiquitinated proteins in brain and spinal cord, and motor axon degeneration in spinal cord (Huang et al., 2011). In addition, overexpressing FUS^{WT} also caused neurodegeneration and ubiquitin-positive inclusions in tissue, though at a later age (Huang et al., 2011).

Since the identification of mutations in ALS-linked genes, many animal models have been created to advance the understanding of ALS disease pathogenesis. The variability in phenotype severity and onset among models, together with the finding that ablation and elevation of the same gene (and wild-type or mutant versions, as well) result in varying degrees of neurodegeneration and other ALS-like symptoms, highlights the complexity of mechanisms resulting in motor neuron death in ALS.

Mouse crossbreeding studies

Many groups have crossbred either $SOD1^{G93A}$ mice or $parkin^{-/-}$ mice to other mice in attempts to define molecular mechanisms that are involved with the pathology and

phenotypic readout of neurodegenerative diseases (a selection of studies are summarized in Tables 1.4 and 1.5). However, crossbreeding of *SOD1*^{G93A} mice with *parkin*$^{-/-}$ mice is a novel system for studying the effect of parkin loss in an ALS mouse model.

Several studies have used *parkin*$^{-/-}$ mice and mutant Tau or α-synuclein mice in crossbreeding experiments, with varied results. Crossing *parkin*$^{-/-}$ mice to mutant A30P α-synuclein mice (a model of PD) resulted in delayed motor decline and less advanced synucleinopathy compared to A30P α-synuclein mice (Fournier et al., 2009), which can be interpreted as an overall improvement in PD-like symptoms. Another group crossing *parkin*$^{-/-}$ mice to mutant A53T α-synuclein mice showed no change in the pathology or behavior of A53T α-synuclein mice (which is hyperactivity due to aberrant dopaminergic neurotransmission and early lethality) (von Coelln et al., 2006), suggesting that the onset and progression of the lethal phenotype due to A53T α-synuclein overexpression are independent of parkin activity. Crossing *parkin*$^{-/-}$ mice to mice with α-synuclein mutated at both A53T and A30P resulted in severe mitochondrial damage, but no histopathological or motor abnormalities (Stichel et al., 2007), showing that while both parkin and α-synuclein are important for mitochondrial health, these changes in mitochondrial morphology do not necessarily cause motor impairment. It is unclear why there is such inconsistency in the phenotypic differences reported in mice with mutant α-synuclein, but the differences may be due to different promoters used to drive transgene expression, by different integration sites of the transgene, or by differences in levels of transgene expression.

Several studies also report that loss of parkin in *Tau*^{VLW} mice (a model of AD and frontotemporal dementia over-expressing a human chromosome 17 [FTDP-17] mutant

with four tau repeats with mutations G272V, P301L, and R406W) creates a more severe phenotype with memory deficits, showing that parkin contributes to maintenance of cellular health in *Tau*^{VLW} mice (Guerrero et al., 2009; Menendez et al., 2006; Rodrıguez-Navarro et al., 2008). Additionally, one group reported that loss of parkin in *Tau*^{VLW} mice produced less prominent motor impairments, and that alternative compensatory mechanisms can be activated in the absence of parkin (Navarro et al., 2008). From these studies, it appears that loss of parkin in *Tau*^{VLW} mice increases memory deficits but decreases motor impairments via compensatory pathways, adding to the complexity of many pathways that may be involved in disease pathogenesis.

SOD1^{G93A} mice are the most commonly used ALS mouse model in SOD1-related research, and have been crossbred to many diverse mutant mice. As might be expected, loss of proteins that normally help regulate glutamate transmission, neuroinflammation, and oxidative stress result in a more severe ALS-like phenotype in *SOD1*^{G93A} mice (Apolloni et al., 2013; Lerman et al., 2012; Pardo et al., 2006; Vargas et al., 2011). On the other hand, two studies suggest that retrograde signaling could be involved in delaying onset of symptoms in *SOD1*^{G93A} mice, though the mechanisms of impaired or increased retrograde transport seem at odds (Perez-Garcia and Burden, 2012b; Teuchert et al., 2006). Most notably, a study crossing *SOD1*^{G93A} mice with Dorfin overexpressing mice saw an amelioration in the *SOD1*^{G93A} mouse phenotype and reduced accumulation of mutant SOD1 in spinal cord tissue (Sone et al., 2010). Dorfin is an E3 ligase reported to recognize mutant SOD1 and facilitate its proteasomal degradation (Niwa et al., 2002), though the ubiquitination linkage and degradation mechanism were not studied. This

report suggests that Dorfin decreases the amount of mutant SOD1 in spinal cords resulting in cytoprotection and an improved ALS-like phenotype in *SOD1*^{G93A} mice.

1.4 Summary and organizational overview

Protein misfolding is intimately linked to the pathogenesis of several neurodegenerative diseases, but the molecular mechanisms that regulate clearance of these misfolded proteins are not well understood. Misfolded SOD1 is implicated in both fALS and sALS (Perez-Garcia and Burden, 2012b). Deficient UPS degradation was found in the *SOD1*^{G93A} mouse model (Cheroni et al., 2009; Cheroni et al., 2005) and overexpression of mutant SOD1 causes proteasomal inhibition and motor neuron-selective cell death (Urushitani et al., 2002), indicating that mutant SOD1 toxicity could be due to failure of the UPS. This raises the possibility that aggregated mutant SOD1 is cleared via the alternate aggresome-autophagy pathway. Our hypothesis is supported by evidence that *SOD1*^{G93A} mice display increased autophagosome formation (Prudencio and Borchelt, 2011), and that mutant SOD1 undergoes aggresome formation (Johnston et al., 2000) and autophagic clearance (Kabuta et al., 2006; Shi et al., 2010). In addition, ubiquitin was found to mediate mutant SOD1 aggregation in ALS mouse models (Polymenidou and Cleveland, 2011). K63-linked polyubiquitination associates with perinuclear inclusions containing mutant SOD1 (Tan et al., 2008), but the E3 ligase responsible for this polyubiquitination and the mechanisms underlying aggregated mutant SOD1 clearance have not yet been identified. In these studies, we found that parkin selectively interacts with mutant SOD1 and promotes its clearance by the aggresome-

autophagy pathway. Using mutant SOD1 as the primary model, my dissertation work examined the role of parkin-mediated K63 polyubiquitination in recruitment of misfolded SOD1 to aggresomes for autophagic clearance, and also demonstrated the importance of parkin's interaction with mutant SOD1 in cell viability.

In Chapter 2, I detail a novel role for parkin in regulating misfolded SOD1 degradation by the aggresome-autophagy pathway. I found that parkin mediates K63-linked polyubiquitination of mutant SOD1 in cooperation with the Ubc13/Uev1a E2 conjugating complex. Knockdown of parkin expression resulted in increased mutant SOD1 levels and decreased ubiquitination of mutant SOD1, showing a role for parkin in regulating mutant SOD1 turnover. I also show that parkin is required for targeting misfolded SOD1 to aggresomes, which are substrates for autophagy and are degraded by the autophagy-lysosome system. In addition, I found that parkin mediates cytoprotection against misfolded SOD1-induced toxicity.

In Chapter 3, I used *parkin*$^{-/-}$/*SOD1*^{G93A} double mutant mice as an innovative model for testing the functional significance of parkin-mediated clearance of mutant SOD1. I found that parkin loss prolongs lifespan and improves motor performance in *SOD1*^{G93A} transgenic mice. Loss of parkin may trigger a compensatory increase in other protein processing mechanisms such as chaperones, demonstrating cross-talk between cellular quality control systems.

Chapter 4 describes the significance of my research and directions for future studies. Understanding the role of parkin-mediated regulation of misfolded proteins will advance our understanding of the molecular mechanisms of neurodegenerative diseases such as ALS.

1.5 Figures and Tables

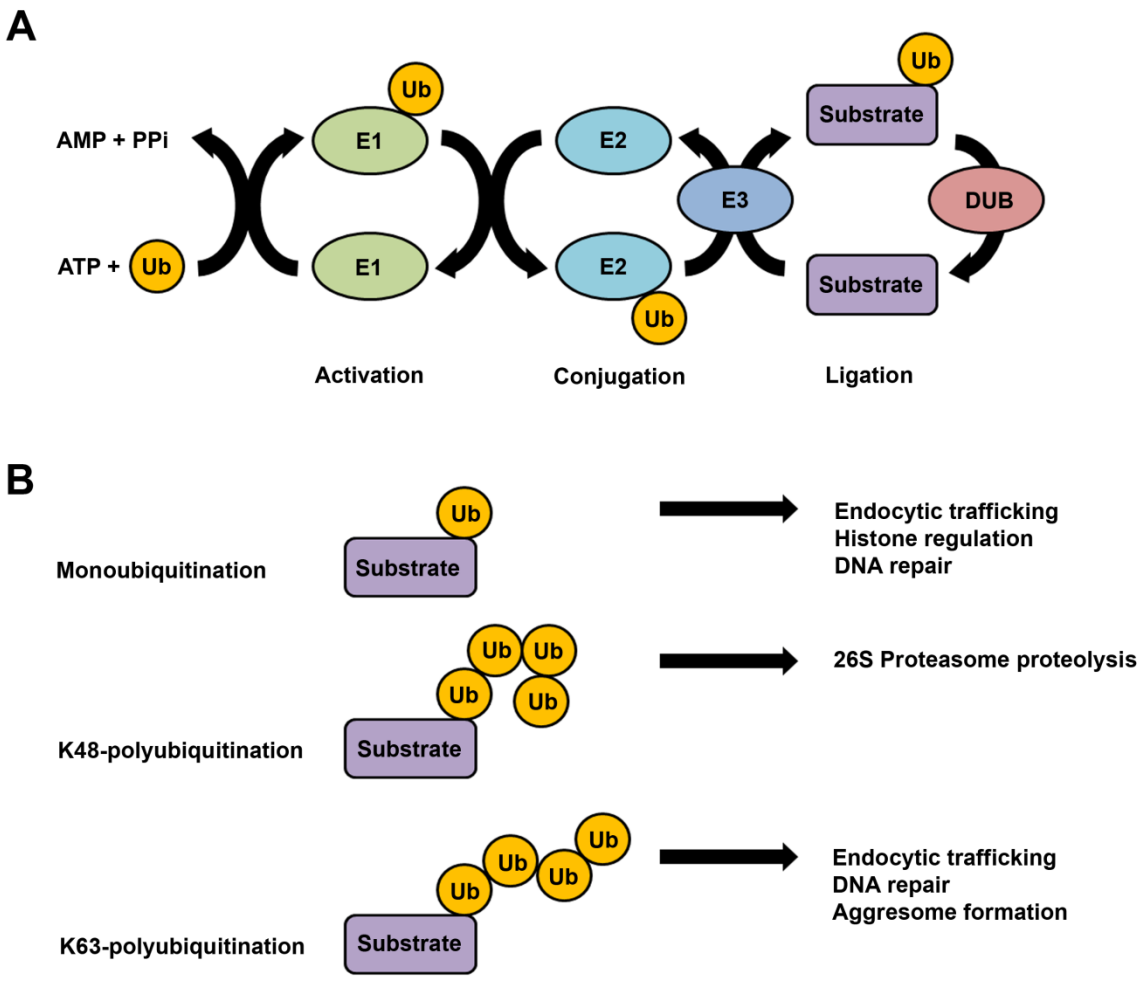

Figure 1.1. The ubiquitin system mediates signaling in cellular processes. **(A)** The ubiquitination cascade requires the ATP-dependent coordination of E1-activating, E2-conjugating, and E3-ligating enzymes that result in attachment of ubiquitin to a lysine residue on a substrate protein. Polyubiquitination occurs by subsequent attachment of ubiquitins to internal lysine residues on ubiquitin. Deubiquitinating enzymes (DUBs) cleave ubiquitin from substrates and are important for ubiquitin chain disassembly. **(B)** Different modes of ubiquitination signal for various cellular processes. Monoubiquitination serves as a signal for endocytic trafficking, histone regulation, and

DNA repair. K48-linked polyubiquitin chains signal for proteasomal degradation, while K63-linked polyubiquitin chains signal for endocytic trafficking, DNA repair, and aggresome formation in targeting substrates for the autophagy-lysosomal degradation pathway.

Table 1.1. Neuropathological hallmarks in neurodegenerative diseases

Disease	*Hallmarks*	*Ubiquitinated aggregate*	*Cell type, localization*	*Affected regions*	*Ref.*
PD	Intracytoplas-mic LBs	LB (α-syn)	Neurons (cytoplasm)	Substantia nigra, brainstem, cortex	(Houlden and Singleton, 2012; Moore et al., 2005)
ALS	Aggregated proteins (*e.g.*,SOD1, FUS, TDP-43)	BB (neurofila-ment)	Motorneurons (cytoplasm, nucleus)	Cortex, bulbar, spinal cord	(Cozzolino et al., 2012; Strong et al., 2005)
AD	Extracellular Aβ/intracellular neurofibrillary tangles	Tangles (Tau)	Neurons (cytoplasm)	Transentorhi-nal, limbic, neocortex	(Braak and Braak, 1991; Braak and Braak, 1995; de Vrij et al., 2004)
HD	Inclusion bodies	Polygluta-mine expanded huntingtin	Neurons (cytoplasm, nucleus)	Striatum, cerebral cortex, subcortical white matter	(Ross and Tabrizi, 2011; Rubinsztein , 2002)

Abbreviations: Aβ, β-amyloid; α-syn, alpha-synuclein; BB, Bunina body; FUS, fused in sarcoma; LB, Lewy body; SOD1, Cu/Zn superoxide dismutase 1; TDP-43, TAR-DNA binding protein 43

Table 1.2. Genes associated with risk of amyotrophic lateral sclerosis

Locus	*Protein (Gene)*	*Inheritance*	*Protein function*	*Ref.*
ALS1	Cu/Zn superoxide dismutase 1 (*SOD1*)	AD, AR	Antioxidant	(Rosen et al., 1993)
ALS2	Alsin (*ALS2*)	AR	Rho guanine nucleotide exchange factor	(Hadano et al., 2001)
ALS3	Unknown	AD	Unknown	(Hand et al., 2002)
ALS4	Senataxin (*SETX*)	AD	RNA/DNA helicase	(Chen et al., 2004)
ALS5	Spatacsin (*SPG11*)	AR	Transmembrane protein	(Hentati et al., 1998)
ALS6	Fused in sarcoma (*FUS*)	AD	RNA binding protein, DNA repair, exon splicing	(Kwiatkowski et al., 2009)
ALS7	Unknown	AD	Unknown	(Sapp et al., 2003)
ALS8	Vesicle-associated membrane protein-associated protein B (*VAPB*)	AD	Vesicular trafficking	(Nishimura et al., 2004)
ALS9	Angiogenin (*ANG*)	AD, sporadic, susceptibility	Ribonuclease	(Greenway et al., 2004)
ALS10	TAR DNA binding protein 43 (*TARDBP*)	AD	Transcriptional repressor, splicing regulation	(Kabashi et al., 2008)
ALS11	*FIG4/SAC3*	AD, sporadic, susceptibility	Lipid phosphatase	(Chow et al., 2009)
ALS12	Optineurin (*OPTN*)	AD, AR	Ocular tension, membrane and vesicle trafficking	(Maruyama et al., 2010)
ALS13	Ataxin 2 (*ATXN2*)	Sporadic, susceptibility	Unknown	(Elden et al., 2010)

ALS14	Valosin-containing protein (*VCP*)	AD	ATP-binding protein, vesicle transport and fusion	(Johnson et al., 2010)
ALS15	Ubiquilin 2 (*UBQLN2*)	X-linked	Ubiquitination, degradation	(Deng et al., 2011)
ALS16	Sigma non-opioid intracellular receptor 1 (*SIGMAR1*)	AR	Endoplasmic reticulum chaperone	(Al-Saif et al., 2011)
ALS-FTD1	Unknown	AD	Unknown	(Hosler et al., 2000)
ALS-FTD2	Chromosome 9 open reading frame 72 (*C9ORF72*)	AD, sporadic	Unknown	(Vance et al., 2006)

Abbreviations: AD, autosomal dominant; AR, autosomal recessive

Table 1.3. Therapeutic approaches in ALS and lifespan outcomes

Therapeutic Approach	*Lifespan outcome*	*Hypothesized Mechanism*	*Ref.*
Riluzole in human ALS patients	Increase lifespan by 2-3 mo	Blocks the presynaptic release of glutamate	(Miller et al., 2012)
Inject rapamycin in *SOD1*^{G93A} mice	Decrease lifespan by 19 d	Activation of apoptotic pathway	(Zhang et al., 2011)
Inject trehalose in *SOD1*^{G93A} mice	Increase lifespan by 32 d in males, 15 d in females	Induction of MTOR-independent autophagy	(Castillo et al., 2013)
MitoQ in *SOD1*^{G93A} mice	Increase lifespan by 6-7 d	Mitochondria-targeted antioxidant	(Miquel et al., 2014)
Lithium and valproic acid in *SOD1*^{G93A} mice	Increase lifespan by 19 d	Inhibitors of glycogen synthase kinase-3 (GSK-3) and histone deacetylases, anti-manic	(Feng et al., 2008)
Galactooligosaccharides (GOS) in *SOD1*^{G93A} mice	Increase lifespan by 13 d	Improves the absorption of B vitamins in colon, lowers level of neurotoxic serum homocysteine	(Song et al., 2013)
Dichloroacetate (DCA) in *SOD1*^{G93A} mice	Increase lifespan by 9 d	Orphan drug that improves mitochondrial redox status via stimulation of the pyruvate dehydrogenase complex activity	(Miquel et al., 2012)
Inject recombinant human Hsp70 in *SOD1*^{G93A} mice	Increase lifespan by 9 d	Increased heat shock response toward cellular stress	(Gifondorwa et al., 2007)
SOD1 G93A vaccine in *SOD1*^{G37R} mice	Increase lifespan by 30 d	Immunization against misfolded SOD1	(Urushitani et al., 2007)

Inject *SOD1*^{G93A} mice with clodronate liposome and bone marrow transplantation of microglia expressing SOD1 WT	Increase lifespan by 11 d	Replacement of toxic mutant SOD1-expressing microglia, improved microenvironment	(Lee et al., 2012a)
Cross *SOD1*^{G93A} mice with Dorfin overexpressing mice	Increase lifespan by 10 d	Dorfin E3 ligase promotes proteasomal degradation of mutant SOD1	(Sone et al., 2010)
Cross *SOD1*^{G93A} mice with *HDAC6*$^{-/-}$ mice	Increase lifespan by 10 d	Decreased α-tubulin deacetylation and improved axonal transport	(Taes et al., 2013)
Cross *SOD1*^{G86R} mice with *Beclin1*$^{+/-}$ mice	Increase lifespan by 14 d	Reduce deregulated levels of autophagy, compensatory mechanisms	(Nassif et al., 2014)

Table 1.4. Summary of selected *parkin*$^{-/-}$ mouse crosses

Cross	*Outcome*	*Hypothesized Mechanism*	*Ref.*
APP$_{swe}$ (K670N and M671L)	less plaques, apoptosis (better)	Compensatory increase in Hsp70, CHIP, macroautophagy	(Perucho et al., 2010)
Expanded huntingtin (*R6/1*)	More severe behavioral abnormality, fewer htt inclusions (worse)	Compensatory autophagy, increase in GSH and LC3-II, decrease Hsp70	(Rubio et al., 2009)
hA30P *α-syn*	Delayed motor decline, less advanced synucleinopathy (better)	Compensatory increase in GSH	(Fournier et al., 2009)
hm^2 *α-syn* (A30P and A53T)	Altered mitochondrial integrity, no motor or histopathological abnormalities (no change)	Changes in mitochondrial morphology do not cause functional impairments	(Stichel et al., 2007)
hA53T *α-syn*	No change in onset or progression of phenotype	α-synuclein induces neurodegeneration independently from parkin E3 activity	(von Coelln et al., 2006)
Tau^{VLW} (G272V, P301L, and R406W)	Memory and exploratory deficits, aggregates in neurons (worse)	Speculate increased Hsp70, GFAP, reduced CHIP	(Guerrero et al., 2009)
Tau^{VLW}	Decreased motor impairments (better)	Less DAT, altered DA metabolism	(Navarro et al., 2008)
Tau^{VLW}	Abnormal behavior, decreased DA neurons in midbrain, plaques (worse)	Less CHIP and Hsp70, more p62, decreased GSH	(Rodrıguez-Navarro et al., 2008)
Tau^{VLW}	Abnormal behavior, decreased DA neurons in midbrain, plaques (worse)	Decreased GSH (oxidative stress), reduced Hsp70 and CHIP	(Menendez et al., 2006)

Table 1.5. Summary of selected *SOD1^{G93A}* mouse crosses

Cross	*Outcome*	*Hypothesized Mechanism*	*Ref.*
Dorfin	Decreased mutant SOD1 protein in spinal cord, increased lifespan (better)	Reduced mutant SOD1 accumulation due to proteasomal degradation	(Sone et al., 2010)
Histone deacetylase 6 deletion (*HDAC6$^{-/-}$*)	Increase lifespan by 10 d (better)	Decreased α-tubulin deacetylation and improved axonal transport	(Taes et al., 2013)
Cramping1 (*Cra1*)	Slowed decline in motor activity and body weight, increased survival (better)	Impaired dynein function and impaired retrograde transport may be beneficial	(Teuchert et al., 2006)
Synaptic receptor tyrosine kinase (*MuSK*)	Delayed onset, reduced muscle denervation, no change in survival (better)	Increased retrograde signaling that promotes differentiation and stabilization of motor nerve terminals	(Perez-Garcia and Burden, 2012a)
Phospholipase C delta 1 deletion (*PLCδ1$^{-/-}$*)	Delayed symptom onset, increased lifespan (better)	Loss of PLCδ1-induced excitotoxicity, increased anti-apoptotic PIP_2	(Staats et al., 2013)
Cyclophilin D deletion (*CypD$^{-/-}$*)	Normal mitochondrial morphology, suppression of motor neuron death, despite disease progression and survival being unaffected (same)	Loss of mitochondrial Ca^{2+} buffering capacity does not contribute to fatality	(Parone et al., 2013)
Glutamate-cysteine ligase modifier subunit deletion (*GCLM$^{-/-}$*)	Decreased lifespan, increased oxidative stress (worse)	Reduced total glutathione, increased oxidative stress	(Vargas et al., 2011)

P2X7 receptor deletion (*P2X7*$^{-/-}$)	Increased motor neuron loss, pro-inflammatory markers (worse)	Neuroinflammation, decreased microglia response	(Apolloni et al., 2013)
Astrocyte glutamate transporter (*GLT1*$^{+/-}$)	Increased rate of motor decline, motor neuron loss (worse)	Glutamate neurotoxicity	(Pardo et al., 2006)
Copper chaperone for SOD1 (*CCS*)	Accelerated neurological deficits, increased mitochondrial load of SOD1 G93A (worse)	Increased CCS changes reduction potential and disulfide bond formation of SOD1 G93A, deleterious protein-protein interactions	(Son et al., 2007)
Galectin-3 deletion (*Gal-3*$^{-/-}$)	More rapid progression, earlier death (worse)	Increased neurotoxic/pro-inflammatory load on neurons	(Lerman et al., 2012)

Chapter 2: Parkin protects against misfolded SOD1 toxicity by promoting its aggresome formation and autophagic clearance

Abstract

Mutations in Cu/Zn superoxide dismutase (SOD1) cause autosomal dominant amyotrophic lateral sclerosis (ALS), a devastating neurodegenerative disease with no effective treatment. Despite ample evidence indicating involvement of mutation-induced SOD1 protein misfolding and aggregation in ALS pathogenesis, the molecular mechanisms that control cellular management of misfolded, aggregation-prone SOD1 mutant proteins remain unclear. Here, we report that parkin, an E3 ubiquitin-protein ligase which is linked to Parkinson disease, is a novel regulator of cellular defense against toxicity induced by ALS-associated SOD1 mutant proteins. We find that parkin mediates K63-linked polyubiquitination of SOD1 mutants in cooperation with the UbcH13/Uev1a E2 enzyme and promotes degradation of these misfolded SOD1 proteins by the autophagy-lysosome system. In response to strong proteotoxic stress associated with proteasome impairment, parkin promotes sequestration of misfolded and aggregated SOD1 proteins to form perinuclear aggresomes, regulates positioning of lysosomes around misfolded SOD1 aggresomes, and facilitates aggresome clearance by autophagy. Our findings reveal parkin-mediated cytoprotective mechanisms against misfolded SOD1 toxicity and suggest that enhancing parkin-mediated cytoprotection may provide a novel therapeutic strategy for treating ALS.

2.1 Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease characterized by degeneration of upper and lower motor neurons (Andersen, 2006), leading to progressive muscle atrophy, paralysis, and death (Rothstein, 2009; Wijesekera and Leigh, 2009). An estimated 90% of ALS cases are sporadic (sALS), and the remaining 10% of cases are familial (fALS) (Rothstein, 2009). Dominantly inherited, missense mutations in the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) were the first identified causative genetic defects for fALS (Andersen, 2006; Rosen et al., 1993; Rothstein, 2009). Ample evidence indicates that the genetic mutations induce SOD1 protein misfolding and aggregation and that SOD1 mutants trigger ALS pathogenesis through a toxic gain-of-function rather than a loss of its enzymatic activity (Galaleldeen et al., 2009; Gaudette et al., 2000; Schmidlin et al., 2009). SOD1 can also become misfolded in sALS due to oxidative damage which causes it to aggregate and form inclusions (Fischer et al., 2004; Gerstner et al., 2008; Hough et al., 2004; Rakhit et al., 2004), similarly to mutant SOD1 (Gruzman et al., 2007; Guareschi et al., 2012). In addition, we have identified SOD1 oxidative damage and aggregation in Alzheimer (AD) and Parkinson disease (PD) brains (Choi et al., 2005). Together, these findings support the involvement of SOD1 protein misfolding in the pathophysiology of ALS and other neurodegenerative diseases and highlight the importance of understanding the molecular mechanisms governing cellular management and removal of misfolded SOD1 proteins.

The ubiquitin-proteasome system (UPS) is a major intracellular protein degradation pathway for eliminating misfolded proteins. Previous studies have identified several E3 ubiquitin-protein ligases that ubiquitinate mutant SOD1 proteins and target

them for degradation by the proteasome (Choi et al., 2004b; Niwa et al., 2002; Urushitani et al., 2004). However, mutant SOD1 proteins have been shown to directly impair proteasome function (Crippa et al., 2010a; Crippa et al., 2010b; Sau et al., 2007), and increasing evidence has implicated the involvement of proteasome dysfunction in the pathogenesis of ALS (Bendotti et al., 2012; Tashiro et al., 2012). The molecular mechanisms that regulate misfolded SOD1 processing and clearance under the conditions of proteasomal impairment remain poorly understood.

Parkin is an E3 ubiquitin-protein ligase whose loss-of-function mutations cause autosomal recessive, early-onset PD (Kitada et al., 1998) devoid of Lewy bodies, a pathological hallmark of PD. We have previously shown that, in response to proteasomal impairment, parkin catalyzes K63-linked polyubiquitination of PD-linked DJ-1 L166P mutant protein and targets misfolded DJ-1 to aggresomes (Olzmann et al., 2007). Parkin has been shown to exert cytoprotective action against proteotoxic stress induced by mutant proteins associated with several neurodegenerative diseases (Petrucelli et al., 2002; Rosen et al., 2010; Tsai et al., 2003). However, whether parkin has a cytoprotective role against ALS-linked mutant SOD1 remains unknown.

Though SOD1 mutant proteins have been reported to undergo both proteasomal degradation (Niwa et al., 2002) and autophagy (Kabuta et al., 2006; Tan et al., 2008), it is unclear what molecular machinery signals for the processing and clearance of mutant SOD1. Misfolded proteins are normally processed by efficient protein quality control systems such as molecular chaperones and the UPS (Goldberg, 2003). However, when these systems become impaired or overwhelmed, misfolded proteins form small, toxic oligomers (Olzmann and Chin, 2008; Olzmann et al., 2008). Aggresome formation is

another cellular defense mechanism that sequesters toxic misfolded proteins and facilitates bulk disposal via lysosome-dependent macroautophagy (hereafter referred to as autophagy) (Iwata et al., 2005; Kopito, 2000; Olzmann et al., 2008). Aggresomes are thought to be cytoprotective and are hypothesized to serve as a foci for autophagic degradation (Kopito, 2000). Autophagic machinery is localized to aggresomes (Iwata et al., 2005; Yamamoto et al., 2006), and inhibition of autophagy blocks aggresome clearance (Fortun et al., 2003; Ravikumar et al., 2002), suggesting that aggresomes are recognized and cleared by autophagic machinery in a process called the aggresome-autophagy pathway.

Various lines of evidence suggest that mutant SOD1 could undergo aggresome formation and clearance by autophagy, though the details of this process are largely unknown. Under proteasome impairment, mutant SOD1 was reported to associate with K63-linked polyubiquitin (Tan et al., 2008), a signal associated with facilitating selective clearance of inclusions via autophagy, though the E3 ligase was not identified. In addition, misfolded SOD1-containing inclusions were found in the perinuclear area (Johnston et al., 2000), showing similar localization and morphology to aggresomes. Here, we report that parkin promotes sequestration of ALS-linked, misfolded SOD1 into aggresomes, regulates lysosomal clustering around SOD1 aggresomes, and facilitates clearance of mutant SOD1 via autophagy. Our results demonstrate a cytoprotective role for parkin in K63-linked polyubiquitination and subsequent autophagic clearance of misfolded SOD1.

2.2 Results

Parkin protects cells against misfolded SOD1-induced toxicity

To determine whether parkin has a role in cellular defense against misfolded SOD1-induced toxicity, we examined the effects of exogenous parkin on cell viability of human neuroblastoma SH-SY5Y cells expressing wild-type SOD1, ALS-linked SOD1 A4V, or G93A mutant proteins in the absence or presence of proteasome inhibition by MG132. Measurement of the extent of cell death using the lactate dehydrogenase (LDH) release assay (Fig. 2.1A) revealed that expression of SOD1 A4V or G93A mutant, but not SOD1 WT, induced cytotoxicity, which was exacerbated by MG132 treatment, consistent with previous reports (Anandhan et al., 2014; Aquilano et al., 2003; Ghadge et al., 2013; Joyce et al., 2015; Kitamura et al., 2014; Rojas et al., 2014). We found that expression of exogenous parkin significantly reduced the cytotoxicity induced by SOD1 A4V or G93A mutants under both basal and MG132-treated conditions (Fig. 2.1A). Similar results were also obtained in independent experiments that assessed the effects of exogenous parkin on vulnerability of SH-SY5Y cells to mutant SOD1-mediated cell death using apoptotic nuclear morphology analysis (Fig. 2.1B and C). Together, these data indicate a cytoprotective role of parkin against misfolded SOD1-induced toxicity.

Parkin mediates K63-linked polyubiquitination of mutant SOD1, but not wild-type SOD1

As a first step to determine the mechanisms underlying parkin-mediated cytoprotection against misfolded SOD1 toxicity, we performed co-immunoprecipitation experiments to

examine the interaction of parkin with wild-type or mutant SOD1 in SH-SY5Y cells. We found that immunoprecipitation of Myc-tagged parkin using anti-Myc antibody co-precipitated SOD1 A4V and G93A mutants, but not SOD1 WT (Fig. 2.2A), indicating a selective interaction of parkin with mutant SOD1 in cells. To test whether parkin and mutant SOD1 interact directly, we performed *in vitro* binding assays using purified recombinant proteins. We found that GST-tagged parkin but not the GST control selectively bound His-tagged SOD1 A4V and G93A, but not SOD1 WT (Fig. 2.2B). Together, these results demonstrate a specific interaction between parkin and mutant SOD1 both *in vivo* and *in vitro*.

Next, we performed *in vitro* ubiquitination analyses with purified recombinant proteins to examine the ability of parkin to ubiquitinate wild-type or mutant SOD1 in the presence of UbcH7, UbcH8, or UbcH13/Uevla E2 ubiquitin-conjugating enzymes which have been shown to facilitate parkin E3 ubiquitin-protein ligase activity (Olzmann et al., 2007). We found that parkin selectively ubiquitinated SOD1 A4V and G93A mutants, but not SOD1 WT, in cooperation with UbcH13/Uev1a, but not with UbcH7 or UbcH8 (Fig. 2.2C). To determine the specific type and ubiquitin linkage of parkin-mediated mutant SOD1 ubiquitination, we performed additional *in vitro* ubiquitination experiments using ubiquitin mutants Ub-K29, Ub-K48, or Ub-K63 (which permit only formation of K29-, K48-, or K63-linked polyubiquitin chains, respectively) and Ub-K0 (which is unable to form polyubiquitin chains due to the mutation of all its lysines to arginines). The results showed that parkin mediated polyubiquitination rather than multi-monoubiquitination of SOD1 A4V and G93A mutants and that parkin-mediated mutant SOD1 polyubiquitination occurred via the K63-linkage (Fig. 2.2D).

We then performed *in vivo* ubiquitination assays to investigate the role of parkin in regulation of wild-type or mutant SOD1 ubiquitination in cells. We found that expression of exogenous parkin promoted ubiquitination of SOD1 A4V and G93A mutants, but not SOD1 WT, under both control and MG132-treated conditions in SH-SY5Y cells (Fig. 2.3A). Through the use of ubiquitin mutants Ub-K0, Ub-K48, and Ub-K63, we showed that parkin predominantly promoted K63-linked polyubiquitination of SOD1 A4V and G93A mutants in cells (Fig. 2.3B and C), consistent with our *in vitro* ubiquitination results (Fig. 2.2D). Furthermore, we found that shRNA-mediated depletion of endogenous parkin in SH-SY5Y cells resulted in decreased ubiquitination of SOD1 A4V and G93A mutants (Fig. 2.4A), indicating that endogenous parkin is required for ubiquitinating mutant SOD1 in cells.

Parkin promotes mutant SOD1 protein degradation by the autophagy-lysosome system

We next studied the effect of parkin depletion on steady-state levels of wild-type and mutant SOD1 proteins using quantitative Western blot analyses. We found that parkin depletion resulted in significant increases in the steady-state levels of SOD1 A4V and G93A mutants but not that of SOD1 WT protein (Fig. 2.4B and C), suggesting that parkin may have a role in selectively regulating mutant SOD1 protein degradation. To examine this possibility, we performed pulse-chase analyses to determine whether parkin affects turnover of wild-type and mutant SOD1 proteins (Fig. 2.5A to D). As reported previously (Borchelt DR, 1995; Johnston et al., 2000; Kabuta et al., 2006; Nakano R, 1996), SOD1 A4V (Fig. 2.5A and C) and G93A (Fig. 2.5A and D) mutants were less

stable and had shorter half-lives than that of SOD1 WT (Fig. 2.5A and B). We found that expression of exogenous parkin significantly accelerated the turnover rates of SOD1 A4V and G93A mutants but not that of SOD1 WT protein (Fig. 2.5A to D), indicating that parkin selectively promotes degradation of mutant SOD1 in cells.

We then used proteasome, lysosome, and autophagy inhibitors to determine which protein degradation system is involved in mediating parkin-regulated mutant SOD1 degradation (Fig. 2.5E to J). Consistent with the ability of parkin to promote mutant SOD1 degradation (Fig. 2.5A to D), expression of exogenous parkin significantly reduced the steady-state levels of SOD1 A4V (Fig. 2.5G and H) and G93A (Fig. 2.5I and J) mutants but not that of SOD1 WT protein (Fig. 2.5E and F). The parkin-induced degradation of SOD1 A4V and G93A mutants was blocked by the autophagy inhibitor 3MA or the lysosome inhibitor chloroquine (CQ), but not by the proteasome inhibitor MG132 (Fig. 2.5G to J). These results provide evidence supporting that parkin promotes mutant SOD1 protein degradation via the autophagy-lysosome system but not the proteasome.

Parkin promotes misfolded SOD1 aggresome formation upon proteasome impairment

To further investigate the mechanisms by which parkin protects against misfolded SOD1 toxicity, we performed immunofluorescence confocal microscopic analysis to examine the effects of parkin on the localization of wild-type or mutant SOD1 in SH-SY5Y cells. We found that, under basal conditions, SOD1 WT and SOD1 A4V or G93A mutants

exhibited a diffuse cytoplasmic distribution (Fig. 2.6A and B) and this cytoplasmic distribution was not affected by coexpression of parkin (Fig. 2.6A and B; Fig. 2.7A and B, top panel). Treatment of the cells with the proteasome inhibitor lactacystin (Fig. 2.6C and D) or MG132 (Fig. 2.7A and B, middle panel) caused accumulation of SOD1 A4V and G93A mutants, but not SOD1 WT, in perinuclear inclusions that resemble aggresomes. Additional immunofluorescence labeling experiments confirmed that the mutant SOD1-containing inclusions were indeed aggresomes, because they were surrounded by a cage of the intermediate filament protein vimentin and were enriched with ubiquitin and Hsp70 (Fig. 2.7C and D). Furthermore, the formation of SOD1 A4V and G93A mutant aggresomes was microtubule-dependent, as their recruitment to the perinuclear region was blocked by the microtubule depolymerizing drug nocodazole, leading to accumulation of these misfolded SOD1 proteins in micro-aggregates scattered throughout the cytoplasm (Fig. 2.7A and B, bottom panel). We observed colocalization of parkin with SOD1 mutants in the micro-aggregates as well as in the perinuclear aggresome (Fig. 2.7A and B), indicating that parkin is well positioned to bind and ubiquitinate misfolded SOD1 both before and after their transport to the perinuclear aggresome. Consistent with our finding of K63-linked polyubiquitination of mutant SOD1 by parkin (Figs. 2.2 and 2.3) and our previous report that K63-linked polyubiquitination serves as an aggresome-targeting signal through its interaction with the dynein adaptor protein HDAC6 (Olzmann et al., 2007), we found that mutant SOD1 aggresomes also contain HDAC6 (Fig. 2.7C and D). Quantitative analyses of aggresome formation revealed that expression of exogenous parkin significantly increased the percentage of cells containing mutant SOD1 aggresomes under the conditions of

proteasome inhibition (Fig. 2.6E), indicating a role for parkin in promoting misfolded SOD1 aggresome formation upon proteasome impairment.

Parkin regulates lysosome positioning around misfolded SOD1 aggresomes

We have previously shown that proteasome impairment causes not only aggresome formation but also redistribution of lysosomes to a perinuclear region to cluster around aggresomes (Lee et al., 2011; Olzmann et al., 2007). Given the emerging evidence indicating the importance of lysosome positioning in the control of autophagic degradation (Korolchuk and Rubinsztein, 2011; Korolchuk et al., 2011), we performed immunofluorescence confocal microscopic analysis to determine whether parkin has a role in regulation of lysosome positioning around aggresomes upon proteasome inhibition. In these experiments, lysosomes were visualized by labeling with antibody against the lysosomal membrane protein LAMP2. We observed that in untransfected cells (e.g., Cell 3 in Fig. 2.8A) or aggresome-lacking, mutant SOD1-expressing cells (Cells 2 and 5 in Fig. 2.8A), lysosomes were widely distributed throughout the cytoplasm under the conditions of proteasome impairment. Only a fraction of cells with mutant SOD1 aggresomes were surrounded by lysosome clusters (Cell 4 in Fig. 2.8A), while other, aggresome-containing cells retain the scattered distribution pattern of lysosomes (e.g., Cell 1 in Fig. 2.8A). We found that the percentage of mutant SOD1 aggresomes with lysosome clustering was significantly increased by expression of exogenous parkin (Fig. 2.8A and B), indicating that parkin promotes lysosome positioning around misfolded SOD1 aggresomes.

Parkin facilitates autophagic clearance of misfolded SOD1 aggresomes

In support of aggresomes being substrates for autophagic clearance, we found that SOD1 A4V- and G93A-containing aggresomes often contain the ubiquitin-binding autophagy receptor p62 and autophagosome marker LC3 (Fig. 2.7C and D). To determine whether parkin has a role in regulating clearance of mutant SOD1 aggresomes, we used a "pulse-chase" aggresome clearance assay (Fortun et al., 2003; Wong et al., 2008) in which cells were first treated with lactacystin for 16 h to allow aggresome formation, lactacystin was then removed, and the fate of aggresomes was monitored after a 24-h chase period. We found that the relative levels of remaining aggresomes containing SOD1 A4V or G93A were significantly reduced by expression of exogenous parkin (Fig. 2.9), indicating accelerated clearance of misfolded SOD1 aggresomes by parkin. Addition of the autophagy inhibitor 3MA in the chase medium resulted in a significant increase in the relative levels of remaining aggresomes, whereas activation of autophagy by mTOR inhibitor rapamycin had an opposite effect (Fig. 2.9). Together, these results support that parkin promotes autophagic clearance of misfolded SOD1 aggresomes.

Parkin is required for misfolded SOD1 aggresome formation and neuroprotection against misfolded SOD1 toxicity

Next, we assessed the effect of targeted parkin deletion on mutant SOD1 aggresome formation by performing immunofluorescence confocal microscopic analysis in primary cortical neurons cultured from *parkin*$^{-/-}$ and *parkin*$^{+/+}$ mice. We found that proteasome

inhibition by MG132 caused accumulation of SOD1 A4V and G93A mutants, but not SOD1 WT, in the perinuclear region to form aggresomes in *parkin*$^{+/+}$ neurons (Fig. 2.10A and D). The formation of SOD1 A4V- or G93A-containing aggresomes was abolished by the loss of parkin in *parkin*$^{-/-}$ neurons (Fig. 2.10B and D), indicating that endogenous parkin is required for misfolded SOD1 aggresome formation. Expression of exogenous parkin in *parkin*$^{-/-}$ neurons restored the ability of these neurons to form mutant SOD1 aggresomes (Fig. 2.10C and D), providing further evidence for a critical role of parkin in misfolded SOD1 aggresome formation. Assessment of neuronal vulnerability to mutant SOD1 toxicity revealed that *parkin*$^{-/-}$ neurons were significantly more susceptible than *parkin*$^{+/+}$ neurons to SOD1 A4V- or G93A-induced apoptosis and this pro-apoptotic phenotype of *parkin*$^{-/-}$ neurons was ameliorated by expression of exogenous parkin (Fig. 2.10E). These results indicate that parkin plays an essential role in neuroprotection against misfolded SOD1 toxicity. Together, our data support the idea that aggresome formation is a cytoprotective response against toxic build-up of misfolded and aggregated proteins.

2.3 Discussion

ALS is a devastating neurodegenerative disorder with many known familial forms caused by mutations in SOD1. Others have found that SOD1 mutants form perinuclear inclusion bodies in cells (Johnston et al., 2000), however the significance and the molecular mechanisms required for processing and clearance of these inclusions are unknown. We found that parkin-mediated degradation of SOD1 mutants was cytoprotective. Others have also found that parkin is cytoprotective, particularly against β-amyloid$_{42}$, another

disease-linked misfolded protein (Rosen et al., 2010). In support of the cytoprotective role of parkin against misfolded, aggregated proteins, we show that parkin promotes mutant SOD1 aggresome formation and clearance, and that parkin expression rescued cell death due to misfolded SOD1 overexpression, as well as proteasomal impairment.

K63-linked polyubiquitination is thought to play a role in inclusion body formation (Lim et al., 2006) and may serve as a signal for targeting ubiquitinated substrates for autophagic degradation (Chin et al., 2010; Kirkin et al., 2009). We found that parkin promotes K63-linked polyubiquitination of misfolded, but not wild-type, SOD1, under proteasomal impairment. In addition, we found that parkin works in cooperation with the UbcH13/Uev1a E2-conjugating complex. This finding is consistent with previous reports that under proteasome impairment, parkin preferentially recruits UbcH13/Uev1a (Lim et al., 2013; Olzmann et al., 2007) to facilitate K63-polyubiquitination of substrate proteins.

Consistent with the view that parkin associates with and polyubiquitinates misfolded proteins before their transport to aggresomes (Olzmann et al., 2007), we found that inhibition of microtubule transport, which is essential for aggresome formation (Johnston et al., 1998), caused parkin to colocalize with misfolded SOD1 in small preaggresomal particles (Olzmann et al., 2007; Tan et al., 2008). Parkin was also found to colocalize with mutant SOD1 in perinuclear aggresomes, indicating ubiquitination could also occur after transport to aggresomes. In addition, mutant SOD1-positive aggresomes were almost never observed in *parkin*$^{-/-}$ neurons, showing that parkin is necessary for targeting misfolded SOD1 to the aggresome. These findings are in accord with previous reports showing lack of protein aggregates in *parkin*$^{-/-}$ mouse brain (Goldberg et al.,

2003; Itier et al., 2003) and the lack of Lewy bodies in familial PD patients with mutations in *parkin* (Mori et al., 1998).

Aggresome and autophagosome formation involve the coordination of several proteins such as p62, LC3, Hsp70, LAMP2, and vimentin, which we detected in or surrounding SOD1 aggresomes. In support of the idea that mutant SOD1 aggresomes are substrates for autophagy, we found that parkin promotes K63-linked polyubiquitination of mutant SOD1 and that mutant SOD1 undergoes parkin-mediated clearance by the autophagy-lysosome system after recruitment to aggresomes. This supports the view that mutant SOD1 is recognized and tagged by parkin for degradation via the aggresome-autophagy pathway, a degradation pathway that has been implicated in several neurological diseases including PD and Charcot-Marie-Tooth disease (Lee et al., 2011; Olzmann et al., 2007). Additionally, we found that parkin regulates misfolded SOD1 degradation, as knockdown of parkin increased SOD1 protein levels and reduced ubiquitination of mutant SOD1. Parkin increased the turnover rate of SOD1 mutants but not WT, as shown by pulse-chase, which further supports a selective role for parkin in mutant SOD1 degradation.

Our findings indicate a regulatory role for parkin in targeting misfolded SOD1 to aggresomes and promoting their clearance by the aggresome-autophagy pathway. Parkin mediates K63-linked polyubiquitination, aggresomal accumulation, and autophagy-lysosomal degradation of misfolded SOD1. We also demonstrate a functional significance of parkin's selective interaction with mutant SOD1 in cell viability. These results provide further details in understanding the molecular mechanism of misfolded SOD1 clearance and cytoprotection in neurodegenerative disease.

2.4 Materials and Methods

Expression constructs and antibodies

Conventional molecular biological techniques were used to generate wild-type or mutant SOD1 in the N-terminal S-tag, His-tag, GFP-tag, and Myc-tag expression vectors by subcloning of SOD1 WT and A4V cDNAs (provided by H. Zhu, University of Kentucky) and SOD1 G93A cDNA (provided by D. H. Lee, Seoul Women's University). The construction of N-terminal GFP-, or GST-tagged parkin was previously reported (McKeon et al., 2015). Other expression constructs used in this study include Myc-tagged parkin (provided by T. Suzuki, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), mCherry-tagged parkin (purchased from Addgene, Cambridge, MA), HA-tagged Ub-WT, Ub-K48, Ub-K63 and Ub-K0 (provided by T. Dawson, Johns Hopkins University, Baltimore, MD). The validity of all constructs was confirmed by DNA sequencing. Parkin shRNA construct and non-targeting, control shRNA construct were purchased from OriGene. Antibodies used in this study include anti-SOD1 (FL-154, Santa Cruz), anti-parkin (Cell Signaling), anti-β-actin (clone C4, Millipore), anti-GST (clone B14, Santa Cruz Biotechnologies), anti-ubiquitin (clone P4G7, Abcam), anti-vimentin (Sigma-Aldrich), anti-p62 (BD Biosciences), anti-LAMP2 (clone H4B4, Iowa Developmental Studies Hybridoma Bank), anti-Hsp70 (Stressgen), anti-HDAC6 (Santa Cruz Biotechnologies), anti-LC3 (Sigma-Aldrich), anti-HA (clone 12CA5), anti-Myc (clone 9E10), and anti-S-tag (Abcam). All secondary antibodies were from Jackson ImmunoResearch Laboratories.

Parkin knockout mice and primary neuronal cultures

Parkin knockout (*parkin*$^{-/-}$) and wild-type (*parkin*$^{+/+}$) mice on a coisogenic background (129S4/SvJaeSor) were generated from breeding pairs provided by R. Palmiter, University of Washington (Seattle, WA) (Perez and Palmiter, 2005). Primary cortical neuronal cultures were prepared from *parkin*$^{-/-}$ and *parkin*$^{+/+}$ mouse embryos at embryonic day 18 as we described previously (Giles et al., 2008) and maintained in NeuroBasal Media (Gibco) supplemented with penicillin/streptomycin (Cellgro), B-27 (Gibco), and L-glutamine (Cellgro) for 7 days. For each experiment, at least 20 transfected neurons per condition were randomly selected for quantification. Data is presented as the mean ± SEM from three independent experiments.

Cell transfection and co-immunoprecipitation

SH-SY5Y cells were cultured with DMEM (Gibco) containing 10% FBS (Atlanta Biologicals) and 0.5% penicillin/streptomycin (Cellgro). SH-SY5Y cells and primary cortical neurons were transfected with the indicated cDNA plasmids using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions. Co-immunoprecipitation analyses were performed as previously described (Olzmann et al., 2004) to determine an interaction between Myc-tagged parkin and S-tagged SOD1 WT or mutants. Briefly, cell lysates from transfected SH-SY5Y cells were subjected to immunoprecipitation with anti-Myc antibody, followed by retrieval of protein complexes using protein G-agarose and immunoblotting.

Immunofluorescence confocal microscopy

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% saponin. Cells were subsequently stained with indicated primary and secondary antibodies and processed for immunofluorescence confocal microscopy as we described previously (Lee et al., 2012b). Cell image acquisition was performed using a confocal laser-scanning microscope (Nikon) with 60x oil-immersion objective, and the acquired images were analyzed using Adobe Photoshop CS4 software.

Cell viability and apoptosis assays

Cell viability was assessed by lactate dehydrogenase (LDH) release assays using the LDH Cytotoxicity Kit (Clontech) in accordance with the manufacturer's protocol. For the analysis of apoptosis in SH-SY5Y cells or primary cortical neurons, transfected cells expressing mCherry or mCherry-parkin and GFP or GFP-SOD1 WT or mutants were identified by red and green fluorescence, respectively. Apoptotic cell death was determined by morphological assessment of nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies) as described (Pridgeon et al., 2007). The percentage of transfected cells with nuclear shrinkage, fragmentation, and chromatin condensation was scored for apoptosis. For each experiment, at least 75-100 transfected cells per condition were randomly selected for quantification in a blinded manner. Data is presented as the mean ± SEM from three independent experiments.

Recombinant protein purification and *in vitro* binding assays

His-tagged SOD1 WT or mutants, glutathione S-transferase (GST), and GST-tagged parkin were individually expressed in *E. coli* BL21 or Arctic Express cells and induced with isopropyl β-D-thiogalactopyranoside (IPTG, 0.1mM for GST-tagged proteins or 1mM for His-tagged proteins; Research Products International) as described (Chin et al., 2000). GST-tagged proteins were purified using glutathione-agarose beads as we described (Chin et al., 2002; Kim et al., 2007), and His-tagged proteins were purified using Ni-NTA-agarose beads and dialyzed in a buffer containing 100 μM each of $CuSO_4$ and $ZnSO_4$ as described (Gazdag et al., 2010). *In vitro* binding assays were performed as described (Li et al., 2001) by incubation of GST-tagged parkin or GST immobilized on glutathione-agarose with purified His-tagged SOD1 WT or mutants, and bound proteins were detected by immunoblotting analysis.

***In vitro* and *in vivo* ubiquitination assays**

In vitro ubiquitination assays were performed as previously described (Olzmann et al., 2007). Briefly, purified His-tagged SOD1 WT or mutants and GST-tagged parkin or GST were incubated in reaction buffer (50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 100 mM NaCl, 25 μM $ZnCl_2$, 2 mM dithiothreitol, and 4 mM ATP) containing E1 enzyme (Boston Biochem), E2 enzymes (UbcH7, UbcH8, or UbcH13/Uev1a, Boston Biochem), and ubiquitin WT or ubiquitin mutants (Boston Biochem). After incubating for 3 h at 37°C, the reaction was stopped by adding loading buffer and ubiquitinated SOD1 was detected by immunoblotting with anti-ubiquitin and anti-SOD1 antibodies. *In vivo*

ubiquitination assays were performed as described (McKeon et al., 2015; Wheeler et al., 2002) in SH-SY5Y cells expressing the indicated epitope-tagged SOD1 WT or mutants, parkin, HA vector, and HA-tagged Ub WT or mutants. SOD1 WT or mutant proteins were isolated by immunoprecipitation under denaturing conditions and their ubiquitination status was assessed by immunoblotting with anti-HA antibody.

[^{35}S] Methionine pulse-chase analysis

Pulse-chase experiments were performed as described (Giles et al., 2008; Olzmann et al., 2004) in HeLa cells expressing indicated proteins. Cells were labeled for 1 h in Met/Cys-free medium (Invitrogen) containing 100 µCi of Met/Cys Tran^{35}S-label (MP Biologicals). After extensive washes, cells were incubated with non-radioactive media containing excess Met/Cys for the indicated chase time. Cells were then lysed and equal amounts of proteins from each lysate were subjected to S-protein bead pull-downs, and ^{35}S-labeled SOD1 WT or mutant proteins were detected by autoradiography. Levels of labeled SOD1 were determined by normalizing the intensity of SOD1 band to the corresponding 0 h SOD1 band using densitometry (ImageJ). Data is presented as the mean ± SEM from three independent experiments.

Analysis of steady-state protein levels

For measurement of steady-state protein levels, cells were either untreated or treated for 24 h with protease inhibitor MG132 (20 µM, Sigma), lysosome inhibitor chloroquine (CQ, 100 µM, Sigma), autophagy inhibitor 3-methyl-adenine (3MA, 10 mM, Sigma), or

0.1% dimethylsulfoxide (DMSO, Fisher) as a vehicle control. Cells were then lysed with 1.1% SDS, protein concentrations were determined using a bicinchoninic acid (BCA) Assay Kit (Pierce). Equal amounts of total proteins from each lysate were subjected to SDS-PAGE and immunoblotting using the indicated antibodies. The protein levels of SOD1 WT or mutants were quantified as described (Giles et al., 2008) and then normalized to the corresponding β-actin levels in the same lysates.

Assessment of aggresome formation and lysosome positioning

Transfected SH-SY5Y cells or primary cortical neurons were incubated in the presence or absence of 5 μM lactacystin for 16 h, and then processed for fluorescence confocal microscopy to assess aggresome formation as described previously (Olzmann et al., 2007). At least 75-100 transfected cells per experimental condition were randomly selected and scored for the presence of SOD1-containing aggresome in a blinded manner, and each experiment was repeated at least three times. For analysis of lysosome positioning, the intracellular distribution of lysosomes was visualized by immunostaining with anti- LAMP2 antibody.

Analysis of aggresome clearance

Aggresome clearance analysis was performed as described (Fortun et al., 2003; Wong et al., 2008). In brief, transfected SH-SY5Y cells were first treated with 5 μM lactacystin for 16 h to allow formation of mutant SOD1 aggresomes. After washing with the media to remove lactacystin, one subset of treated cells was immediately processed for

fluorescence microscopy to visualize mutant SOD1 aggresomes, and three parallel subsets of identically treated cells were allowed to recover for 24 h in normal media containing 0.1% DMSO (vehicle), 100 nM rapamycin (Sigma), or 10 mM 3MA (Sigma). Following 24 h recovery, cells were processed for evaluation of remaining aggresomes. Quantification was carried out in a blinded manner from randomly selected, 75-100 transfected cells in each group, and experiments were repeated at least three times.

Statistical analysis

Data were analyzed by unpaired two-tailed Student's *t* test or one- or two-way ANOVA followed by Tukey's post-hoc analysis, and $P < 0.05$ was considered statistically significant. Results are expressed as mean ± SEM from at least three independent experiments.

2.5 Figures and Legends

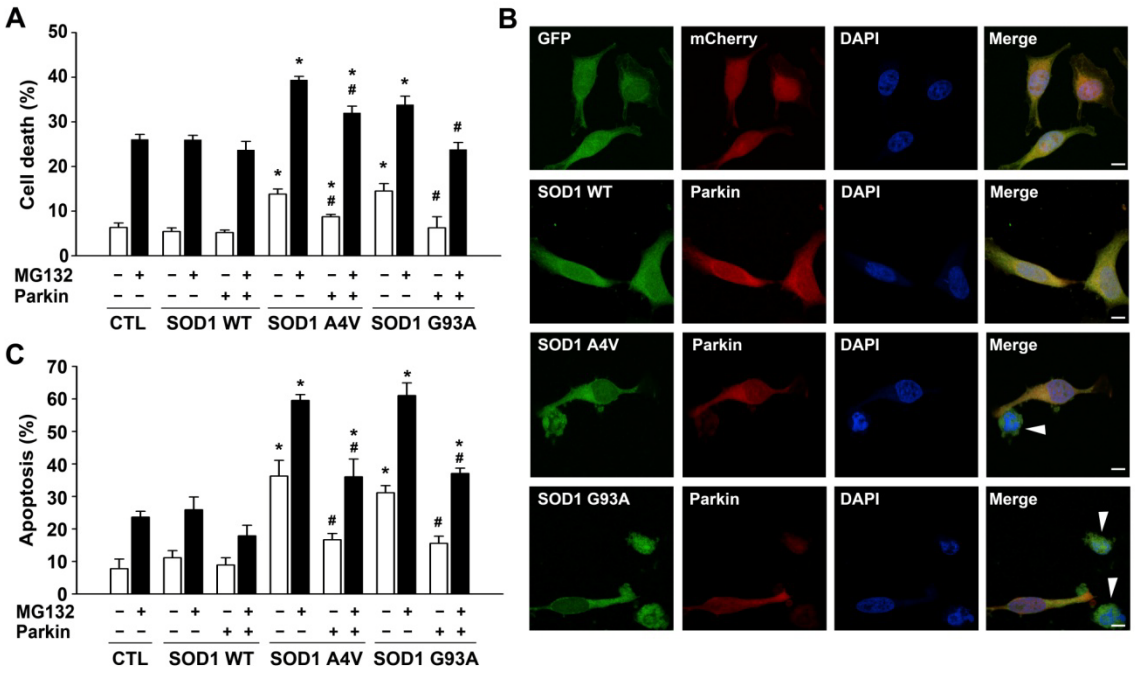

Figure 2.1. Parkin confers cytoprotection against mutant SOD1-induced toxicity. **(A)** SH-SY5Y cells expressing Myc vector control (CTL), Myc-tagged SOD1 WT or mutants and GFP-tagged parkin or GFP were incubated in the absence or presence of 20 μM MG132 for 24 h. The extent of cell death was determined by measuring the amount of LDH released in the culture media and normalized to the total amount of LDH upon cell lysis. Data represent mean ± SEM from three independent experiments. * $P < 0.05$ versus the corresponding Myc vector control or SOD1 WT-expressing cells; # $P < 0.05$ versus the corresponding control cells lacking exogenous parkin, two-way ANOVA with Tukey's post-hoc test. **(B** and **C)** SH-SY5Y cells co-transfected with mCherry or mCherry-tagged parkin and GFP or GFP-tagged SOD1 WT or mutants were incubated in the absence or presence of 20 μM MG132 for 24 h. Fluorescence images (B) show transfected cells identified by GFP (green) and mCherry (red) fluorescence, and nuclear

integrity assessed by DAPI staining (blue) under normal culture conditions. Apoptotic nuclei are denoted with arrowheads. Scale bar = 10 μm. The bar graph (C) shows the extent of apoptosis as the percentage of GFP control (CTL), SOD1 WT or SOD1 mutant-transfected cells with apoptotic nuclear morphology. Data represent mean ± SEM from three independent experiments. * $P < 0.05$ versus the corresponding GFP control or SOD1 WT-expressing cells; # $P < 0.05$ versus the corresponding mCherry control cells lacking exogenous parkin, two-way ANOVA with Tukey's post-hoc test.

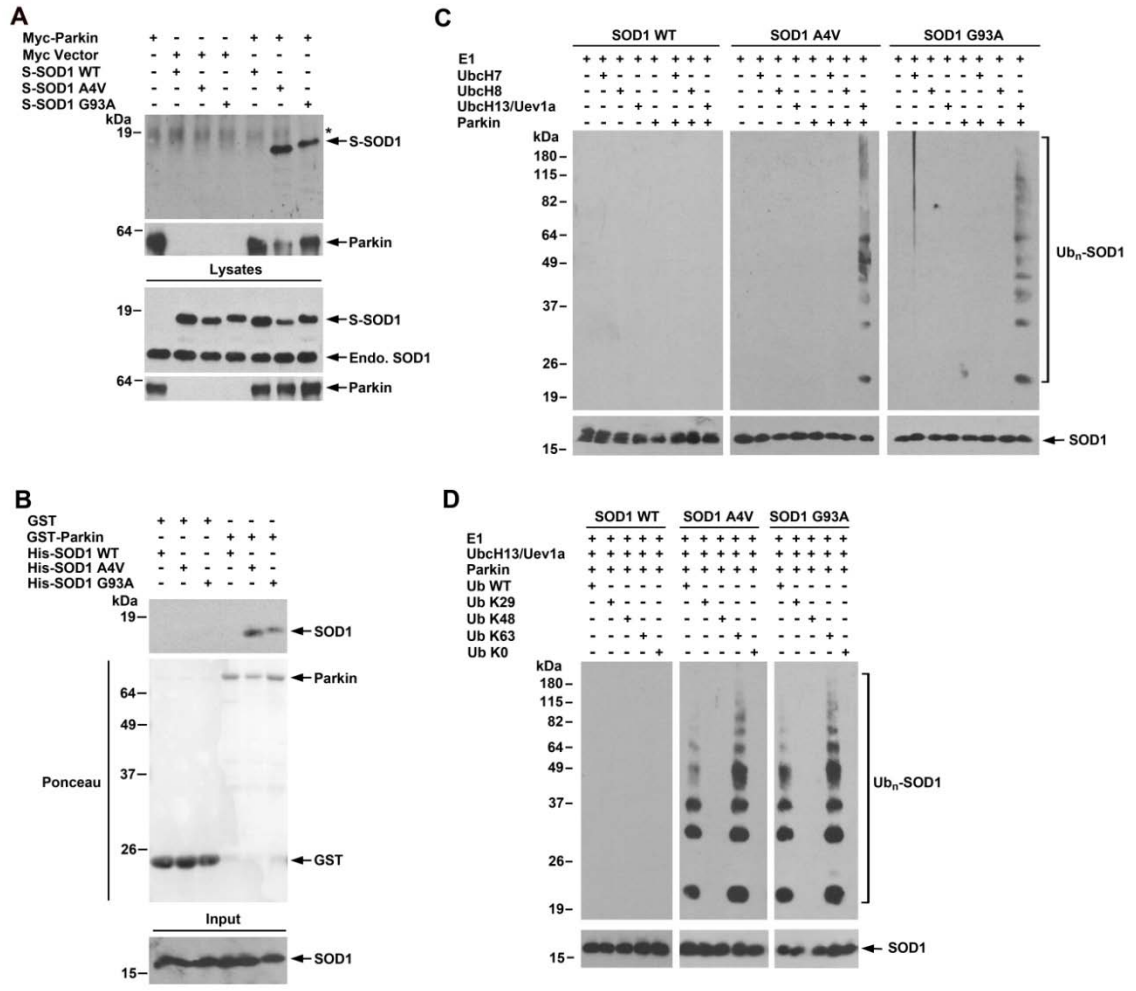

Figure 2.2. Selective interaction and ubiquitination of mutant SOD1 by parkin. **(A)** Co-immunoprecipitation analysis reveals a specific interaction of parkin with SOD1 A4V and G93A mutants, but not SOD1 WT. Lysates from transfected SH-SY5Y cells were subjected to immunoprecipitation with anti-Myc antibody followed by immunoblotting with anti-Myc antibody and anti-SOD1 and antibody, which recognized both S-tagged and endogenous (endo.) SOD1 proteins. * Denotes nonspecific band. **(B)** *In vitro* binding assays were performed by incubation of soluble His-tagged SOD1 WT or mutants (input) with immobilized GST or GST-parkin (shown by Ponceau stain). Analysis of bound proteins by immunoblotting with anti-SOD1 antibody shows direct binding of mutant

SOD1 to parkin. **(C)** *In vitro* ubiquitination analysis of purified SOD1 WT or mutants in the presence of indicated E1, E2 (UbcH7, UbcH8, or UbcH13/Uev1a), GST-parkin or GST, and ubiquitin reveals selective ubiquitination of mutant SOD1 by parkin in cooperation with the UbcH13/Uev1a E2 enzyme. **(D)** *In vitro* ubiquitination analysis of SOD1 WT or mutants in the presence of E1, UbcH13/Uev1a, GST-parkin, and indicated wild-type or mutant ubiquitin shows that *in vitro* polyubiquitination of mutant SOD1 by parkin occurs via the K63-linkage.

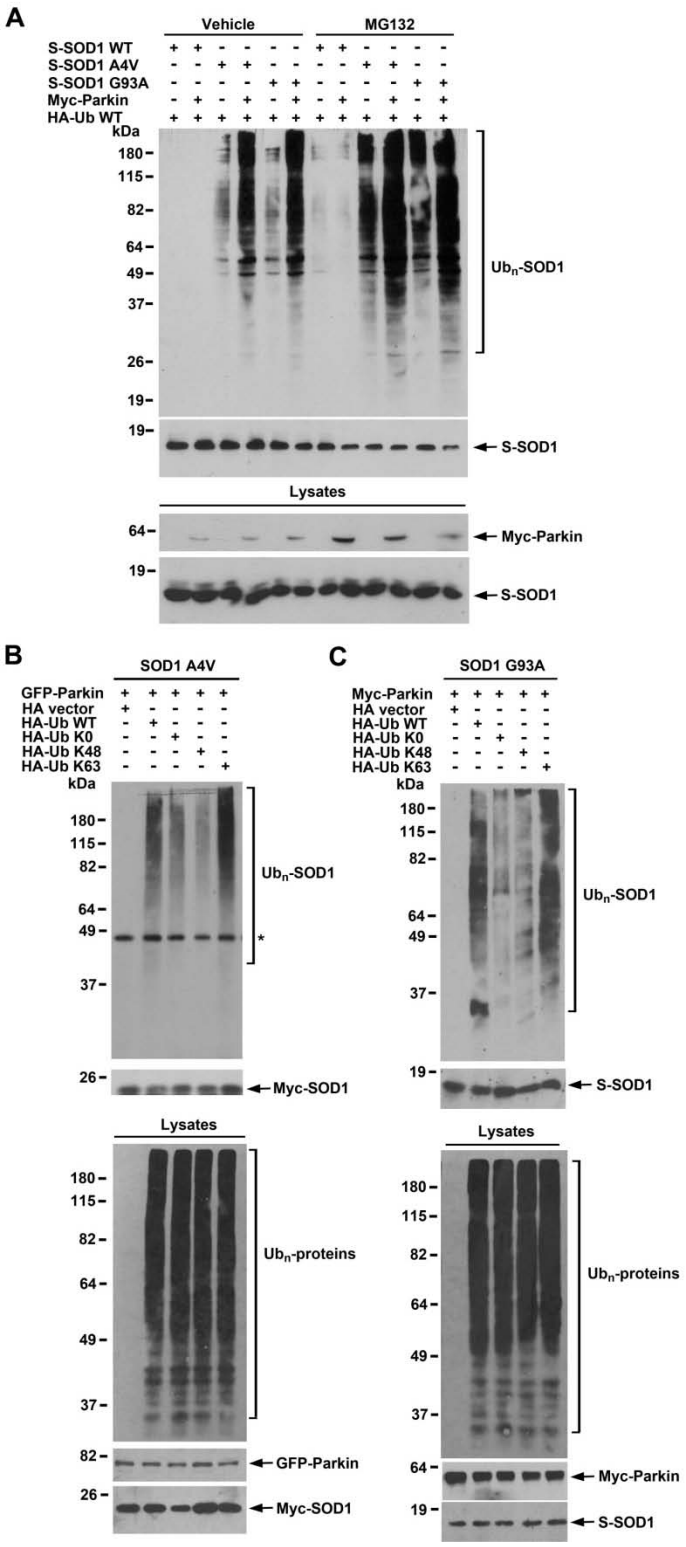

Figure 2.3. Parkin facilitates K63-linked polyubiquitination of mutant SOD1 in cells. **(A)** SH-SY5Y cells expressing indicated S-tagged SOD1 WT or mutants, Myc-tagged parkin

and HA-tagged ubiquitin were treated with vehicle (0.1% DMSO) or 20 μM MG132 for 8 h. *In vivo* ubiquitination of SOD1 WT or mutants was assessed by S-protein bead pulldown under denaturing conditions followed by immunoblotting using anti-HA and anti-S-tag antibodies. Ub_n, polyubiquitin. **(B)** *In vivo* ubiquitination analysis in SH-SY5Y cells expressing Myc-tagged SOD1 A4V, GFP-tagged parkin, and indicated HA-tagged ubiquitin WT or mutants following treatment with 20 μM MG132 for 8 h reveals that parkin preferentially promotes K63-linked polyubiquitination of SOD1 A4V. * Denotes nonspecific band. **(C)** *In vivo* ubiquitination analysis in SH-SY5Y cells expressing S-tagged SOD1 G93A, Myc-tagged parkin, and indicated HA-tagged ubiquitin WT or mutants following treatment with 20 μM MG132 for 8 h shows that parkin preferentially promotes K63-linked polyubiquitination of SOD1 G93A.

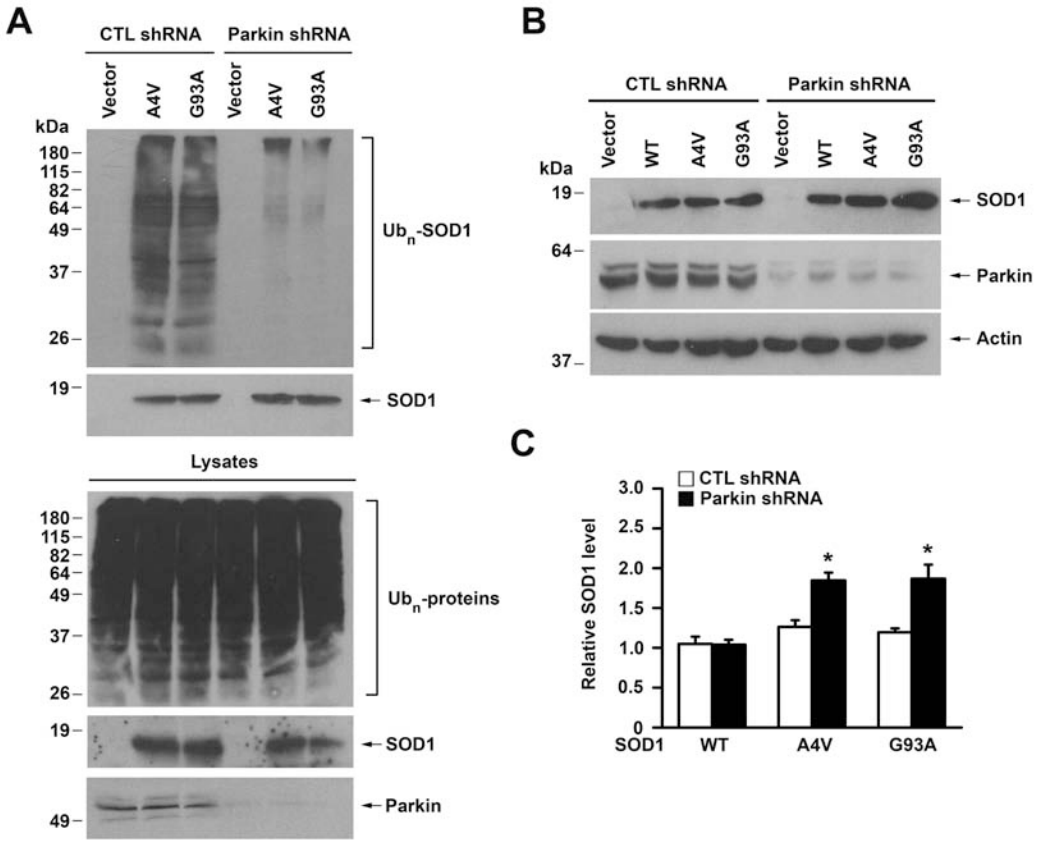

Figure 2.4. Parkin depletion alters ubiquitination and steady-state levels of SOD1 mutants. **(A)** SH-SY5Y cells were transfected with indicated CTL shRNA or parkin shRNA, S-tagged SOD1 mutants, and HA-tagged ubiquitin. *In vivo* ubiquitination of SOD1 mutants was assessed by S-protein bead pulldown under denaturing conditions followed by immunoblotting with anti-HA and anti-S-tag antibodies. Ub_n, polyubiquitin. Depletion of endogenous parkin was confirmed by immunoblotting analysis of cell lysates using anti-parkin antibody. **(B)** Lysates from CTL shRNA- or parkin shRNA-transfected SH-SY5Y cells expressing S-tagged SOD1 WT or mutants or vector control were analyzed by immunoblotting with anti-S-tag, anti-parkin and anti-β-actin antibodies. **(C)** The relative level of SOD1 WT or mutant was normalized to the β-actin level in the

corresponding cell lysate and expressed relative to the normalized SOD1 level in the CTL shRNA-transfected SOD1 WT-expressing cell lysate. Results are shown as mean ± SEM from three independent experiments. * $P < 0.05$ versus the corresponding CTL shRNA-transfected control, unpaired two-tailed Student's *t* test.

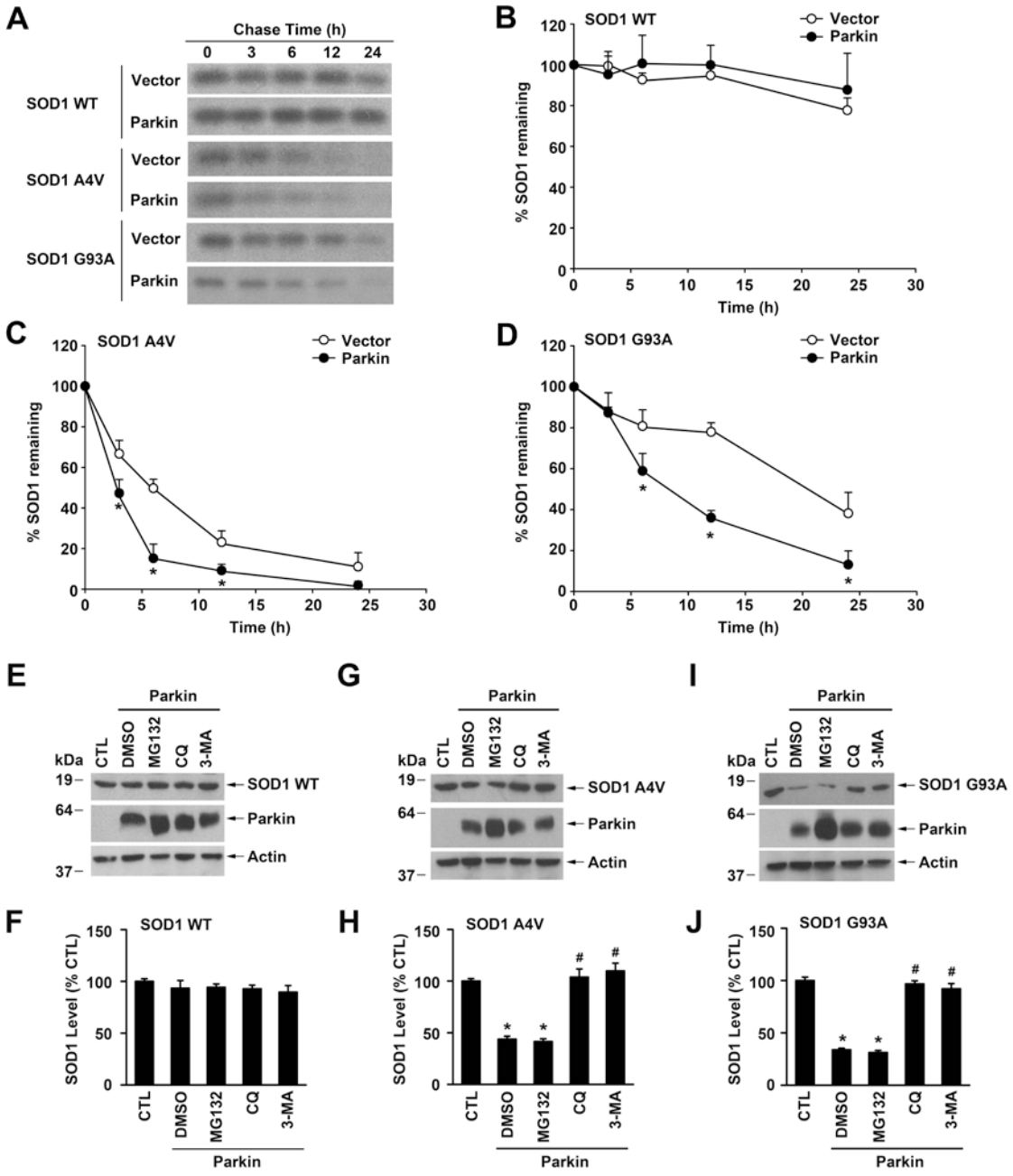

Figure 2.5. Regulation of mutant SOD1 protein degradation by parkin. **(A)** The degradation of S-tagged SOD1 WT, A4V, or G93A in vector- or parkin-transfected HeLa cells was analyzed by [^{35}S]Met/Cys pulse-chase assays. Cell lysates were subjected to S-protein-bead pulldown and ^{35}S-labeled SOD1 was detected by autoradiography. **(B-D)** The protein levels of ^{35}S-labeled SOD1 WT (B), A4V (C), and G93A (D) were quantified

and plotted relative to their corresponding protein levels at 0 h. Data represent mean ± SEM from three independent experiments. * $P < 0.05$ versus the corresponding vector-transfected control, two-way ANOVA with Tukey's post-hoc test. **(E-J)** SH-SY5Y cells transfected with S-tagged SOD1 WT (E), A4V (G), or G93A (I) and Myc-tagged parkin were treated for 24 h with the indicated protein degradation inhibitors or the vehicle DMSO. The corresponding vector-transfected, DMSO-treated cells were used as the control (CTL). Lysates were analyzed by immunoblotting with anti-S-tag, anti-parkin, and anti-β-actin antibodies. The relative level of SOD1 WT (F), A4V (H), or G93A (J) was normalized to the β-actin level in the corresponding cell lysate and expressed as a percentage of the normalized SOD1 level in the corresponding CTL cells. Results are shown as mean ± SEM from three independent experiments. * $P < 0.05$ versus DMSO-treated, Myc vector-transfected control; $^{\#}$ $P < 0.05$ versus the DMSO-treated, Myc-parkin-transfected control, one-way ANOVA with Tukey's post-hoc test.

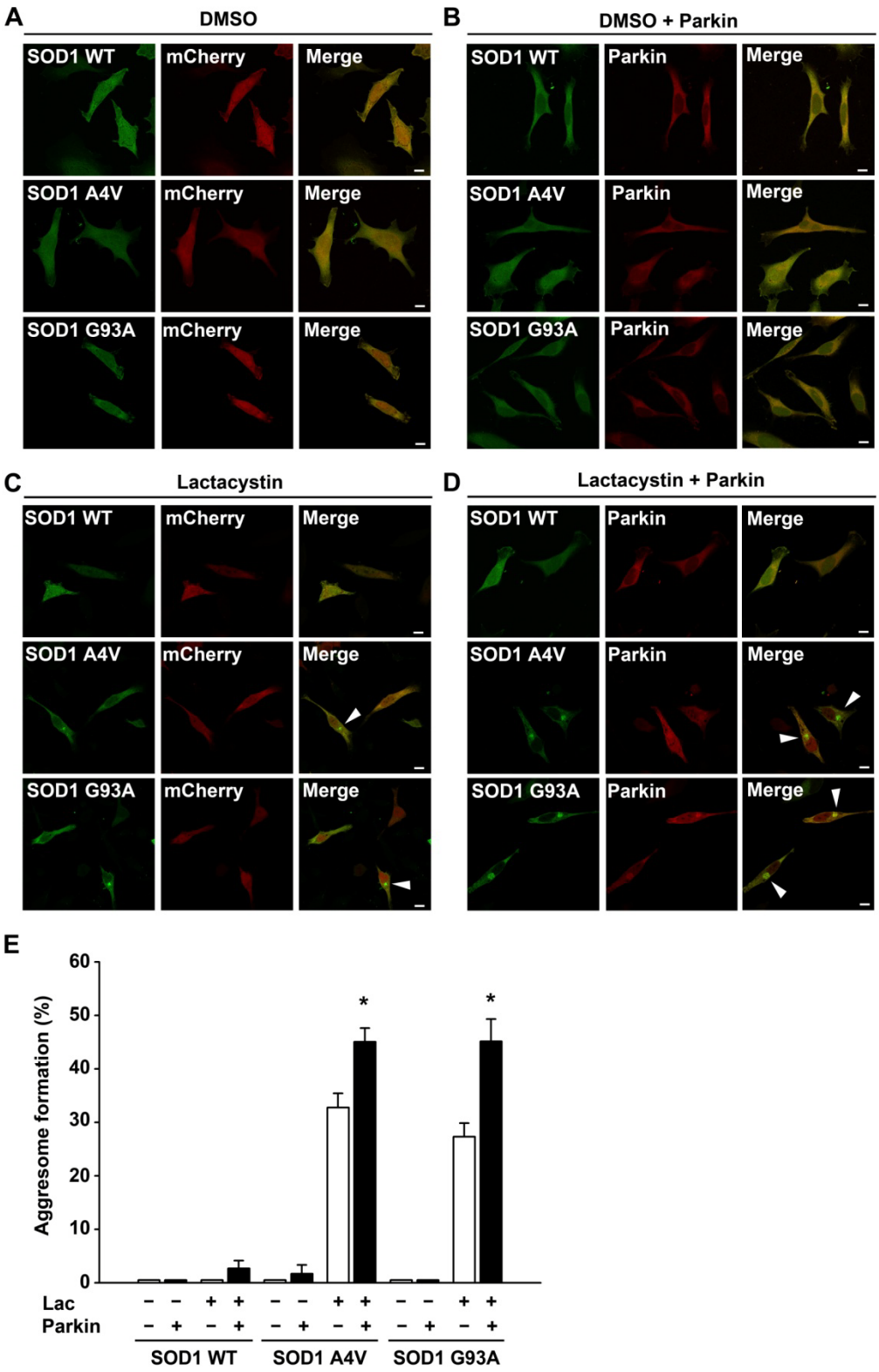

Figure 2.6. Parkin promotes formation of mutant SOD1 aggresomes. **(A-D)** SH-SY5Y cells expressing GFP-tagged SOD1 WT, A4V, or G93A and mCherry or mCherry-parkin

were treated with the vehicle DMSO (A, B) or 5 μM lactacystin (C, D) for 16 h and then imaged by fluorescence confocal microscopy. The presence of perinuclear aggresomes is indicated by the arrowhead. Scale bar = 10 μm. **(E)** Aggresome formation was quantified and expressed as the percentage of SOD1-transfected cells containing SOD1-positive aggresomes. * $P < 0.05$ versus the corresponding mCherry control cells lacking exogenous parkin, unpaired two-tailed student's *t* test.

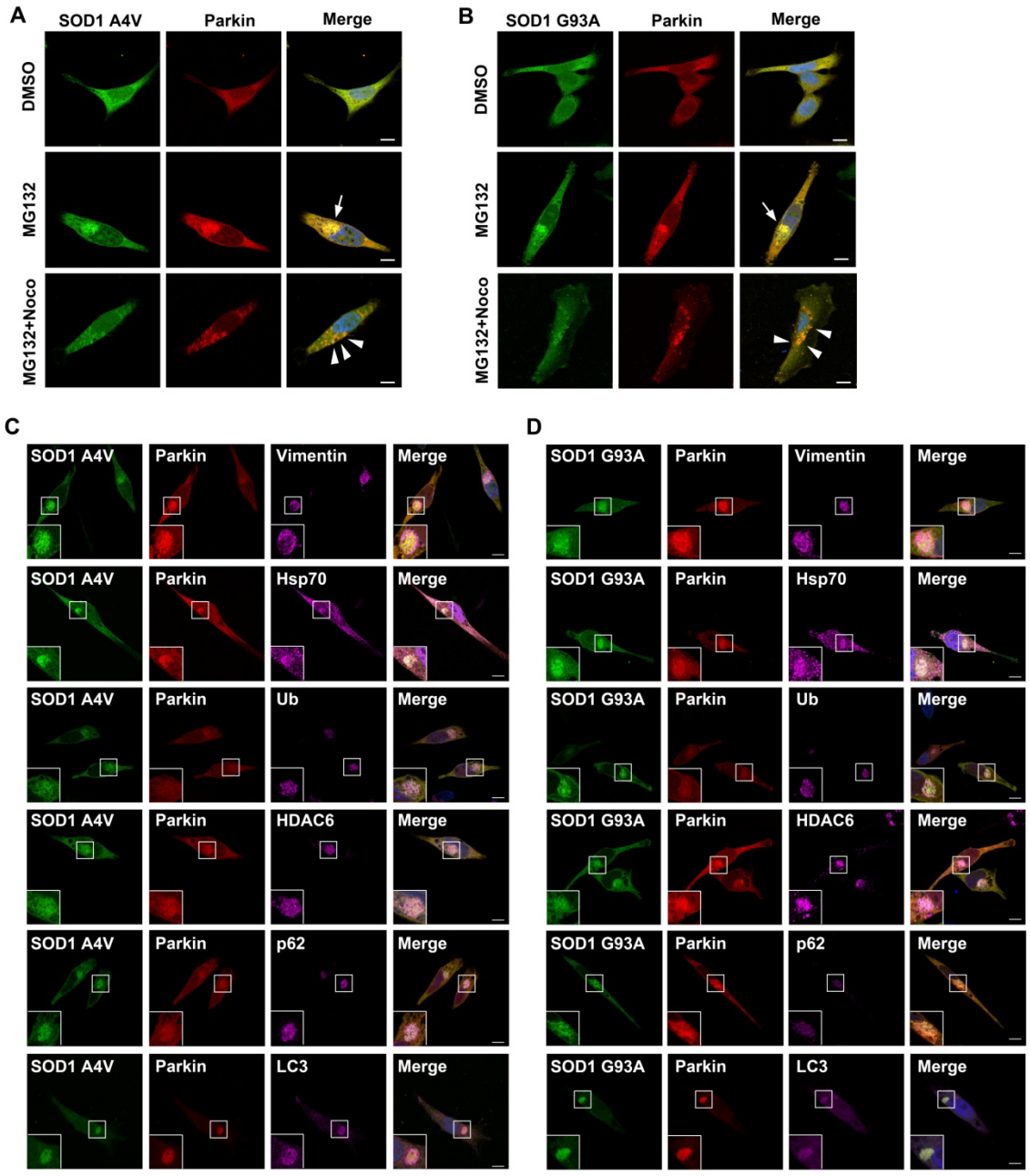

Figure 2.7. Characterization of mutant SOD1 aggresomes by multi-labeling immunofluorescence confocal microscopy. (**A-B**) Mutant SOD1 aggresome formation is microtubule-dependent. SH-SY5Y cells expressing mCherry-parkin and GFP-tagged SOD1 A4V (A) or G93A (B) were incubated with the vehicle DMSO, 5 μM MG132, or 5

μM MG132 plus 5 μg/ml nocodazole (Noco) for 24 h as indicated. Merged channel shows GFP-SOD1 (green), mCherry-parkin (red), and DAPI stained nuclei (blue). MG132 treatment alone resulted in the formation of mutant SOD1-positive perinuclear aggresomes (arrows), whereas MG132 plus nocodazole treatment caused formation of mutant SOD1-positive micro-aggregates (arrowheads) instead of aggresomes. Scale bar = 10 μm. **(C-D)** Immunostaining of SH-SY5Y cells expressing mCherry-parkin and GFP-tagged SOD1 A4V (C) or G93A (D) following treatment with 5 μM MG132 for 24 h using anti-vimentin, anti-Hsp70, anti-ubiquitin, anti-HDAC6, anti-p62, and anti-LC3 antibodies. Merged channel includes GFP-SOD1 (green), mCherry-parkin (red), the indicated markers (purple), and DAPI-stained nuclei (blue). Scale bar = 10 μm.

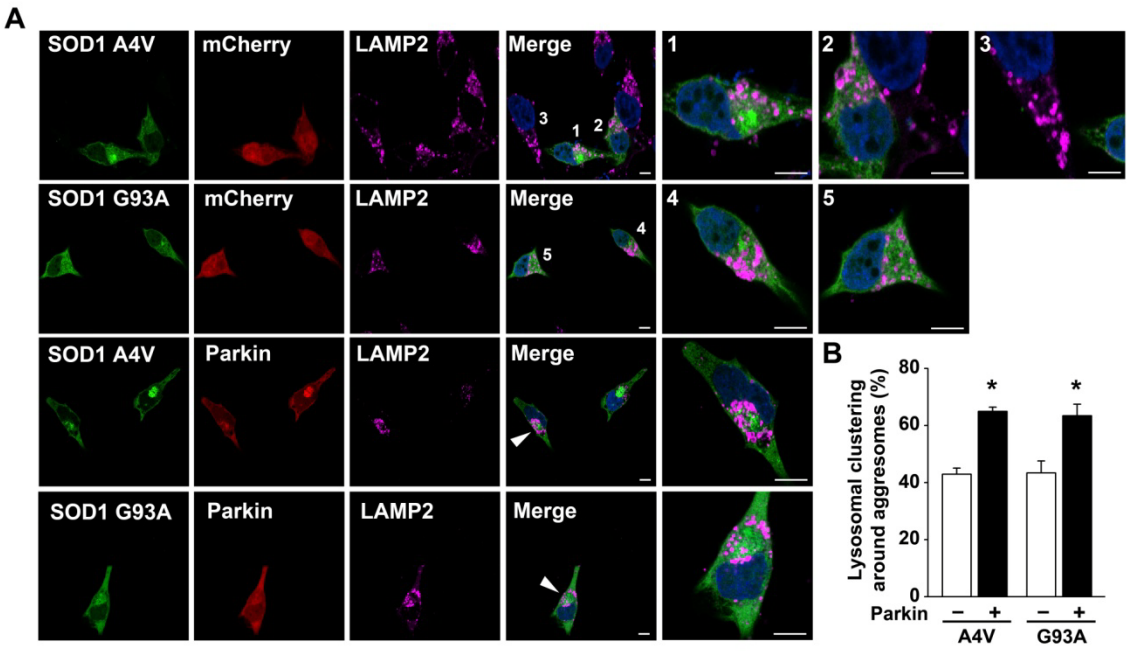

Figure 2.8. Parkin promotes lysosome clustering around mutant SOD1 aggresomes. **(A)** SH-SY5Y cells transfected with mCherry or mCherry-parkin and GFP-SOD1 A4V or G93A were treated with 5 µM lactacystin for 16 h and then processed for immunofluorescence confocal microscopic analysis with anti-LAMP2 antibody to visualize lysosome positioning. Merged channel includes GFP-SOD1 (green), LAMP2-positive lysosomes (purple), and DAPI-stained nuclei (blue). The numbers or arrowheads indicate the cells shown in the enlarged view. Scale bar = 10 µm. **(B)** Quantification of the percentage of aggresomes in transfected cells that have lysosomal clustering around them. * $P < 0.05$ versus the corresponding mCherry control cells lacking exogenous parkin, unpaired two-tailed Student's *t* test.

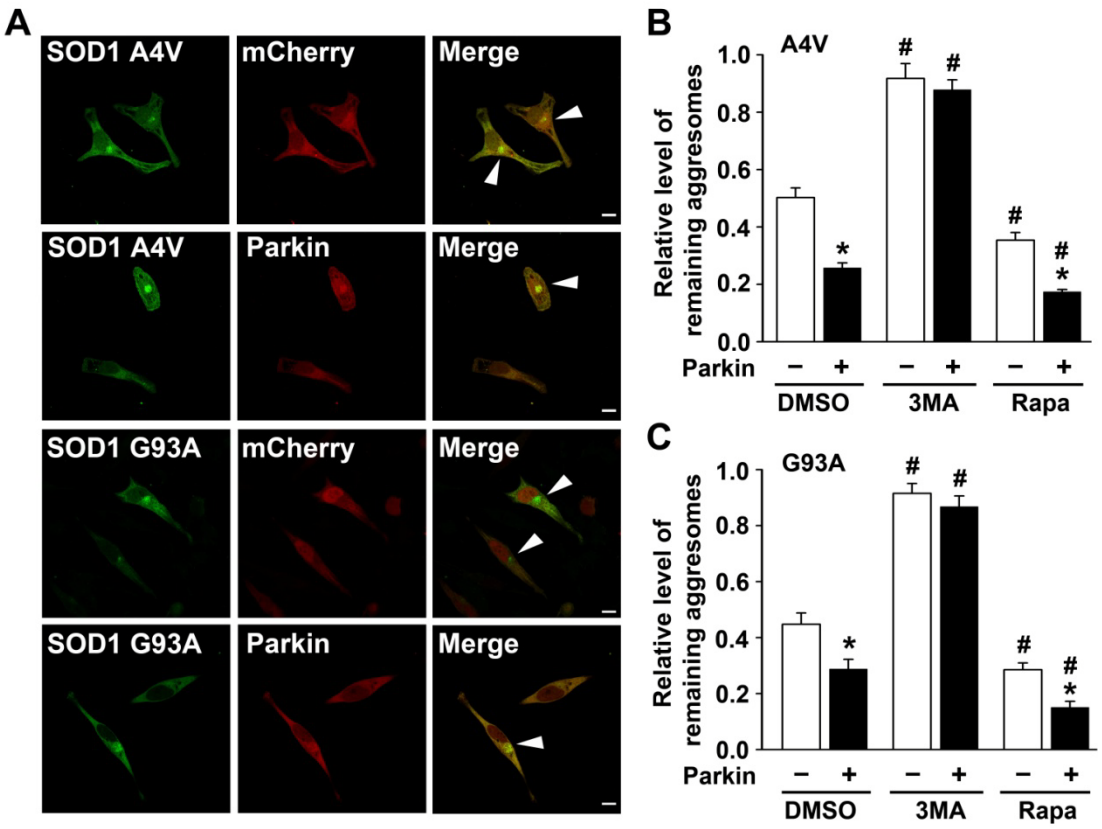

Figure 2.9. Parkin facilitates clearance of mutant SOD1 aggresomes. **(A)** Confocal images of SH-SY5Y cells expressing GFP-tagged SOD1 A4V or G93A with mCherry or mCherry-parkin after treatment with 5 μM lactacystin for 16 h followed by a 24-h chase period in normal culture media. Arrowheads indicate cells with remaining aggresomes. Scale bar = 10 μm. **(B** and **C)** The relative level of remaining aggresomes is determined by quantifying the percentage of cells with remaining aggresomes after a 24-h chase period in normal culture media with DMSO (vehicle), 3MA, or rapamycin (Rapa) and normalized to the percentage of cells with aggresomes formed by the 16-h lactacystin treatment in corresponding cells. * $P < 0.05$ versus the corresponding mCherry-expressing control; # $P < 0.05$ versus the corresponding cells chased in DMSO-containing media, two-way ANOVA with Tukey's post-hoc test.

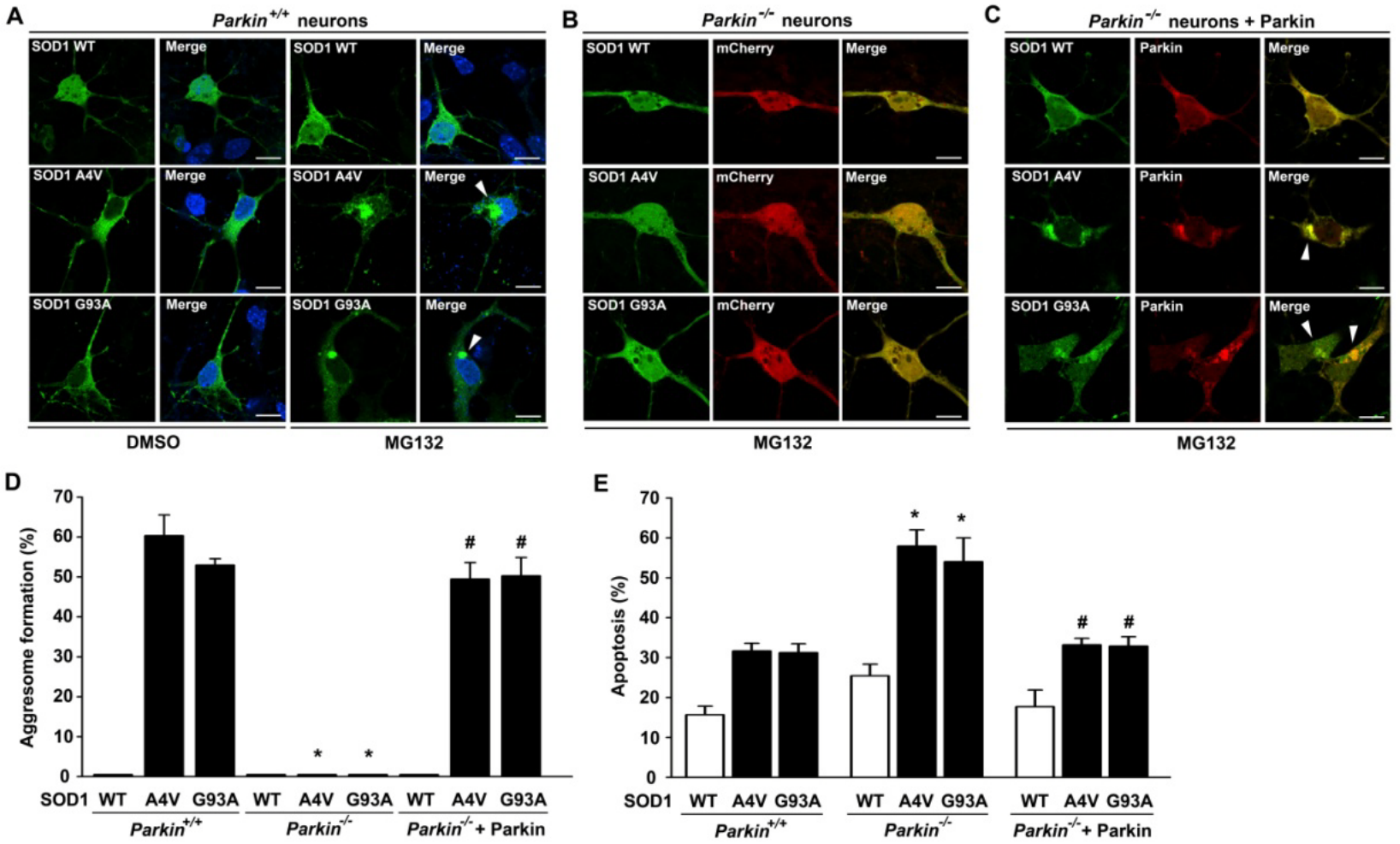

Figure 2.10. Targeted parkin deletion abolishes mutant SOD1 aggresome formation and enhances neuronal vulnerability to mutant SOD1-induced toxicity. **(A-C)** *Parkin*$^{+/+}$ and *Parkin*$^{-/-}$ primary cortical neurons transfected with indicated GFP-tagged SOD1 WT or

mutant and mCherry or mCherry-parkin were treated with the vehicle DMSO or 5 μM MG132 for 24 h as indicated and then imaged by fluorescence confocal microscopy. Aggresomes are indicated by arrowheads. Merged channel in (A) shows GFP-SOD1 (green) and DAPI-stained nuclei (blue). Scale bar = 10 μm **(D)** Aggresome formation was quantified and expressed as the percentage of SOD1-transfected neurons containing SOD1-positive aggresomes. * $P < 0.05$ versus the corresponding *parkin*$^{+/+}$ control; # $P < 0.05$ versus the corresponding mCherry-transfected *Parkin*$^{-/-}$ neurons, two-way ANOVA with Tukey's post-hoc test. **(E)** *Parkin*$^{+/+}$ and *Parkin*$^{-/-}$ primary cortical neurons transfected with indicated GFP-tagged SOD1 WT or mutant and mCherry or mCherry-parkin were treated with 5 μM MG132 for 24 h, and nuclear integrity was assessed by DAPI staining. Apoptosis is expressed as the percentage of SOD1-transfected cells with apoptotic nuclear morphology. Data represent mean ± SEM from three independent experiments. * $P < 0.05$ versus the corresponding control level in *parkin*$^{+/+}$ neurons; # $P < 0.05$ versus the corresponding level in *Parkin*$^{-/-}$ neurons, two-way ANOVA with Tukey's post-hoc test.

Chapter 3: Parkin deletion ameliorates disease phenotype in the *SOD1*^{G93A} mouse model of amyotrophic lateral sclerosis

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal motor neuron disease. Dysfunction in protein quality control machinery is hypothesized to contribute towards ALS pathology, as evidenced by the accumulation of mutant, misfolded Cu/Zn superoxide dismutase (SOD1) in the spinal cord of ALS patients. We previously identified parkin as a cytoprotective E3 ubiquitin-protein ligase that selectively recognizes mutant, but not wild-type, SOD1, and promotes K63-linked polyubiquitination of mutant SOD1 for degradation by the aggresome-autophagy pathway. Here, we studied the role of parkin in *SOD1*^{G93A} transgenic mice, a commonly used mouse model of familial ALS. Surprisingly, in addition to delaying onset of behavioral symptoms and extending lifespan in the *SOD1*^{G93A} mice, loss of parkin also resulted in delayed denervation at neuromuscular junctions. Our study suggests that in a *SOD1*^{G93A} mouse model, loss of parkin activates compensatory pathways that ameliorate ALS-like symptoms.

3.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurological disease characterized by selective degeneration of upper and lower motor neurons, leading to muscle atrophy, weakness, and death, usually within five years of diagnosis (Logroscino et al., 2008; Rothstein, 2009; Wijesekera and Leigh, 2009). It is estimated that 90-95% of ALS cases are sporadic (sALS), with no identified cause, while the remaining 5-10% of cases are hereditary or familial (fALS) (Rothstein, 2009), but both types have indistinguishable pathology. Human genetic studies have revealed that about 20% of fALS cases are caused by mutations in Cu/Zn superoxide dismutase (SOD1) (Andersen, 2006; Rosen et al., 1993), and over 100 ALS-linked mutations within SOD1 have been identified (Abel et al., 2012).

Impairment of protein quality control machinery has been implicated in ALS etiology (Kabashi and Durham, 2006). Mutations in SOD1 such as G93A, as well as oxidized SOD1 WT, cause misfolding of the protein and subsequent cytotoxic SOD1 oligomerization (Bosco et al., 2010; Furukawa and O'Halloran, 2005; Galaleldeen et al., 2009). Accumulation of toxic, misfolded SOD1 can impair proteasome function (Cheroni et al., 2009; Cheroni et al., 2005), which promotes motor neuron-selective cell death in mouse models of ALS (Urushitani et al., 2002). When protein quality control systems such as molecular chaperones and the ubiquitin-proteasome system become damaged or overwhelmed, degradation of aggresomes via lysosome-dependent macroautophagy (hereafter, autophagy) is another cellular defense system (Iwata et al., 2005; Kopito, 2000; Olzmann et al., 2008). The E3 ligase E6-AP has been shown to

suppress mutant SOD1 aggregation (Mishra et al., 2013), and another E3 ligase, Dorfin, is reported to promote proteasomal degradation of mutant SOD1 (Niwa et al., 2002), but the ubiquitin linkage and molecular mechanisms underlying mutant SOD1 aggregate formation and clearance remain largely unknown.

Parkin is an E3 ubiquitin-protein ligase linked to juvenile-onset autosomal recessive Parkinson disease (PD) devoid of Lewy bodies (Kitada et al., 1998). We have previously identified parkin as a selective interactor of misfolded SOD1, promoting K63 polyubiquitin-mediated aggresome formation and subsequent degradation of misfolded SOD1 aggresomes by the autophagy-lysosomal pathway. Parkin is cytoprotective in other models of misfolded protein-induced toxicity, such as mutant APP overexpression in Alzheimer disease (AD) (Hong et al., 2014). Dorfin overexpression was shown to ameliorate the *SOD1*^{G93A} mouse phenotype and reduce levels of accumulated mutant SOD1 in spinal cord (Sone et al., 2010), suggesting a cytoprotective role for E3 ligases in ALS. However, accumulated misfolded proteins found in diseased tissue often consist of aggregates too large to undergo proteasomal degradation (Bence et al., 2001; Bennett et al., 2005), raising the possibility for a bulk-degradation process such as parkin-mediated aggresome-autophagy clearance in these instances.

SOD1^{G93A} mice display shortened lifespan and motor neuron degeneration (Gurney et al., 1994) and are the most commonly used mouse model of ALS. By crossing *SOD1*^{G93A} mice with *parkin*$^{-/-}$ mice, we tested the hypothesis that parkin loss would exacerbate the *SOD1*^{G93A} mouse ALS-like phenotype due to loss of parkin-mediated cytoprotection. Surprisingly, parkin loss significantly delayed disease

progression and increased lifespan in *SOD1*^{G93A} mice. Our findings suggest that loss of parkin may be a beneficial modifier of misfolded SOD1 pathogenesis.

3.2 Results

Parkin and mutant SOD1 interact *in vivo*

We previously described a selective interaction of parkin with mutant, but not wild-type, SOD1 and established a role for parkin-mediated K63 polyubiquitination of mutant SOD1 in aggresome formation and clearance by the autophagy-lysosome pathway. To determine if parkin and mutant SOD1 interact in an *in vivo* mouse model, mouse brain homogenates from nontransgenic control, *SOD1*^{WT} overexpressing transgenic, or *SOD1*^{G93A} overexpressing transgenic mice were subjected to co-immunoprecipitation with anti-parkin antibody and bound protein complexes were eluted from protein G agarose beads (Fig. 3.1). Immunoblotting analysis using anti-SOD1, anti-parkin, and anti-β-actin antibodies determined a specific interaction of parkin with mutant SOD1 G93A. The C4F6 SOD1 antibody is raised against the misfolded SOD1 G93A mutant protein and binds preferentially to misfolded, mutant SOD1 proteins compared to SOD1 WT (Urushitani et al., 2007). Immunoblotting analysis using anti-SOD1 C4F6 antibody confirmed a specific interaction of parkin with misfolded SOD1 G93A and not the wild-type SOD1 protein (Fig. 3.1).

Generation of *parkin*$^{-/-}$*/SOD1*^{G93A} double mutant mice

Since we established an interaction of parkin and SOD1 G93A in mice, we wanted to determine if parkin is protective against misfolded SOD1 *in vivo*. To investigate the

pathogenic role of parkin loss in a *SOD1*^{G93A} transgenic mouse model of ALS, we generated double mutant mice with hemizygous or homozygous deletion of parkin (*parkin*$^{-/-}$/*SOD1*^{G93A}, *parkin*$^{+/-}$/*SOD1*^{G93A}, and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates). Transgenic mice overexpressing human SOD1 G93A were bred with parkin-null mice to establish an F2 generation on the same mixed C57BL/6J and 129S4/SvJaeSor background (Fig. 3.2A). Double mutant progeny were identified using PCR analysis of tail genomic DNA (Fig. 3.2B, C). The expression of SOD1 G93A and parkin were confirmed by immunoblot analysis of brain lysates from transgenic mice using a conformational antibody specific for misfolded SOD1 (anti-SOD1 C4F6) and an anti-parkin antibody (Fig. 3.2D).

Parkin loss ameliorates ALS-like phenotypes in *SOD1*^{G93A} mice

A decrease in weight from the peak body weight is interpreted as a measure of early stages of disease, and is accompanied by hindlimb tremor (Parone et al., 2013). A 10% reduction in body weight was considered the end of the early phase and start of the late phase of disease (Watanabe et al., 2014) (Fig. 3.5A). The late phase of disease is characterized by progressive weight loss, loss of the hindlimb splaying reflex, and paralysis of both hindlimbs (Parone et al., 2013). Loss of parkin in *SOD1*^{G93A} mice prolonged lifespan by about 27 d compared to *parkin*$^{+/+}$/*SOD1*^{G93A} control mice, and a 10 d increase in *parkin*$^{+/-}$/*SOD1*^{G93A} mice (Table 3.1 and Fig. 3.3A). Tremor onset was not significantly different among the three genotypes studied (Table 3.1 and Fig. 3.3B);

however, the duration of tremors was increased in *parkin*$^{-/-}$/*SOD1*^{G93A} mice due to the lengthened lifespan (Table 3.1).

The date at which maximum weight was reached was delayed by about 25 d in *parkin*$^{-/-}$/*SOD1*^{G93A} mice and 5 d in *parkin*$^{+/-}$/*SOD1*^{G93A} mice (Table 3.1). In plotting the raw weights of the mice over time, it is apparent that *parkin*$^{-/-}$/*SOD1*^{G93A} mice have a delay in weight decline especially towards the end stage (Fig. 3.4A). *Parkin*$^{-/-}$/*SOD1*^{G93A} mice maintained an increase in weight until about 131 d, almost 25 days longer than *parkin*$^{+/+}$/*SOD1*^{G93A} mice and 30 days longer than *parkin*$^{+/-}$/*SOD1*^{G93A} mice (Table 3.1 and Fig. 3.4A). The probability of mice reaching maximum body weight over time was also delayed in *parkin*$^{-/-}$/*SOD1*^{G93A} mice (Fig. 3.3C), signifying delay in early disease phase onset.

Total disease duration, as measured by the time between tremor onset until end point (Fig. 3.5A), was increased for *parkin*$^{-/-}$/*SOD1*^{G93A} mice only (Fig. 3.5B). This is due to the increased lifespan without changes in tremor onset. The *parkin*$^{-/-}$/*SOD1*^{G93A} mice averaged a disease duration of 68 ± 6.5 d, with *parkin*$^{+/-}$/*SOD1*^{G93A} mice at 53 ± 6.0 d and *parkin*$^{+/+}$/*SOD1*^{G93A} mice at 47 ± 4.5 d. The early phase of disease progression, considered the time from tremor onset to 10% weight reduction, was delayed by about 17 d in *parkin*$^{-/-}$/*SOD1*^{G93A} mice (Fig. 3.5C). The *parkin*$^{-/-}$/*SOD1*^{G93A} mice had an average early phase duration of 54.4 ± 6.3 d, the *parkin*$^{+/-}$/*SOD1*^{G93A} mice 38.3 ± 4.8 d, and the *parkin*$^{+/+}$/*SOD1*^{G93A} mice 37.6 ± 4.8 d. The average duration of the late phase of disease was not different among the genotypes (Fig. 3.5D). In addition, *parkin*$^{-/-}$/*SOD1*^{G93A} mice maintained a higher body weight at end stage (Fig. 3.5E) (average 19.8 ± 0.7 g compared to 17.1 ± 0.3 g for *parkin*$^{+/-}$/*SOD1*^{G93A} mice and 18.0 ± 0.6 g for *parkin*$^{+/+}$/*SOD1*^{G93A}

mice) and lost less weight over the course of disease (average end stage weight was 84.0 ± 1.3% of maximum weight), compared to *parkin*$^{+/-}$/*SOD1*^{G93A} mice (74.9 ± 1.6% of maximum weight) and *parkin*$^{+/+}$/*SOD1*^{G93A} mice (74.7 ± 2.3% of maximum weight) (Fig. 3.5F).

Parkin$^{-/-}$/*SOD1*^{G93A} mice showed higher latency to falling from the Rotarod compared to both *parkin*$^{+/+}$/*SOD1*^{G93A} mice and *parkin*$^{+/-}$/*SOD1*^{G93A} mice starting at 119-133 d, which continued until 161 d, indicating improved motor performance and delayed disease phenotype (Fig. 3.6A). Similarly, *parkin*$^{-/-}$/*SOD1*^{G93A} mice maintained a higher latency to falling from the inverted grid in the hang test, a measure of grip strength, starting at 91 d and continuing until 154 d (Fig. 3.6B). Interestingly, *parkin*$^{+/-}$/*SOD1*^{G93A} mice show a significant decline in hang test performance compared to *parkin*$^{+/+}$/*SOD1*^{G93A} mice starting at 105 d, which progresses rapidly to failure in the hang test (0 seconds) to death.

Loss of the hindlimb splaying reflex indicates functional paralysis of both hindlimbs, although the animal may still be able to right itself (righting reflex) within 30 s when placed on its side (Solomon et al., 2011). Onset of hindlimb paralysis (determined by observation of hindlimbs being held in a retracted position when lifted by the tail and loss of the hindlimb splaying reflex) was delayed in *parkin*$^{-/-}$/*SOD1*^{G93A} mice by about 23 d (164.3 ± 4.1 d compared to 141.0 ± 2.8 d in *parkin*$^{+/+}$/*SOD1*^{G93A} mice), and delayed about 4 d in *parkin*$^{+/-}$/*SOD1*^{G93A} (145.1 ± 2.5 d) (Table 3.1). There was no significant difference in the duration of paralysis among the three genotypes studied (Table 3.1). In mice aged 138 d, *parkin*$^{+/+}$/*SOD1*^{G93A} and *parkin*$^{+/-}$/*SOD1*^{G93A} mice display loss of the hindlimb splaying reflex and hold both hindlimbs in a retracted

position indicative of paralysis, but *parkin*$^{-/-}$/*SOD1*^{G93A} mice maintain the hindlimb splaying reflex at this age, though tremor is present (Fig. 3.7A). These results indicate disease onset in *parkin*$^{-/-}$/*SOD1*^{G93A} mice, but not to the severity of *parkin*$^{+/+}$/*SOD1*^{G93A} and *parkin*$^{+/-}$/*SOD1*^{G93A} mice at the same age. As expected, nontransgenic control mice are able to extend their hindlimbs fully in the splaying reflex and do not display hindlimb tremor. Quantification of the probability of mice displaying loss of the hindlimb splaying reflex with hindlimb posture limited to a retracted position over time indicates a delay in hindlimb paralysis in *parkin*$^{-/-}$/*SOD1*^{G93A} mice (Fig. 3.7B).

Parkin loss delays muscle denervation and axonal degeneration in *SOD1*^{G93A} mice

Classic pathological hallmarks of ALS model *SOD1*^{G93A} mice include muscle denervation and axonal degeneration (Vinsant et al., 2013). We assessed the denervation of neuromuscular junction (NMJ) endplates from the tibialis anterior hindlimb muscle of double-mutant mice to determine whether the delay in clinical onset of symptoms observed in *parkin*$^{-/-}$/*SOD1*^{G93A} mice corresponded to a delay in NMJ denervation. In presymptomatic 60 d old *parkin*$^{+/+}$/*SOD1*^{G93A} mice, NMJs were about 57% innervated, 14% partially denervated, and 30% fully denervated (Fig. 3.8). A progressive shift toward increased denervation at 110 d of age is seen with about 25% innervated, 15% partially denervated, and 60% denervated endplates at this symptomatic stage (Fig. 3.8). This trend corresponds with the age-dependent, progressive decrease in motor function in *parkin*$^{+/+}$/*SOD1*^{G93A} mice.

In contrast, *parkin*$^{-/-}$/*SOD1*^{G93A} mice of the same ages had significantly more innervated NMJs and fewer denervated NMJs. The percentage of denervated neuromuscular junctions (NMJs) in *parkin*$^{-/-}$/*SOD1*^{G93A} mice was about 5% at 60 d and about 13% at a 110 d (Fig. 3.8B). Similarly, *parkin*$^{-/-}$/*SOD1*^{G93A} mice had about 4% of NMJs partially denervated at 60 d and about 8% partially denervated at 110 d. The percentage of innervated NMJs remained at about 80-90% at both ages of *parkin*$^{-/-}$/*SOD1*^{G93A} mice (Fig. 3.8B). Overall, the percentage of NMJ innervation was not different between 60 d old and 110 d old *parkin*$^{-/-}$/*SOD1*^{G93A} mice, indicating a delay in denervation and partial denervation in these mice. Therefore, loss of parkin, through yet unknown mechanisms, may play an important role for cytoprotection against misfolded SOD1-induced toxicity in a *SOD1*^{G93A} mouse model.

3.3 Discussion

In this study, we examined the effect of parkin loss in an ALS mouse model. We previously showed that parkin overexpression is cytoprotective against misfolded SOD1-induced toxicity and that parkin promotes formation of misfolded SOD1-containing aggresomes which are degraded by the autophagy-lysosome system. Therefore, we hypothesized that parkin suppression would aggravate the behavioral deficits and pathology of *SOD1*^{G93A} mice. Surprisingly, we found that parkin loss ameliorated the phenotypic decline in motor function of *SOD1*^{G93A} mice, including delayed onset of weight loss, hindlimb paralysis, motor performance deficits, and NMJ denervation, which resulted in increased lifespan.

SOD1^{G93A} transgenic mice are reported to have between about 30-50% NMJ denervation at age 60 d and about 50-70% NMJ denervation at 110 d, with motor performance decline, the first indication of clinical disease, beginning around 78 d (Fischer et al., 2004). Our findings support this observation of an age-dependent increase in NMJ denervation with similar percentages. Loss of parkin in *SOD1*^{G93A} mice at both ages resulted in significant decreases in NMJ denervation and corresponding increases in the number of innervated endplates. In fact, the number of innervated, partially denervated, and denervated endplates was not significantly different between 60 d old and 110 d old *parkin*$^{-/-}$/*SOD1*^{G93A} mice, indicating protection against NMJ denervation even at a symptomatic stage of disease. All double mutant mice tested in this study reached endpoint (severe hindlimb paralysis and the inability to right itself when placed on its back within 30 s). Therefore, loss of parkin does not completely prevent NMJ denervation and onset of an ALS-like phenotype, but rather significantly delays its progression. At this point, it is unclear whether the ALS-like phenotype observed in these mice is due solely to denervation of NMJs or an overall loss of motor neurons, or perhaps both. Future experiments exploring pathological changes could study the number of toluidine blue-stained motor axons from ventral roots of these mice. Motor neuron disease has been described as a "dying back" motor neuropathy, where distal axonal degeneration at the NMJ occurs early on in disease, before neuronal degeneration and symptom onset (Fischer et al., 2004; Frey et al., 2000). Insight into the health and number of motor axons in these mice could help determine if decreased motor neuron death contributes to the delayed disease phenotype in *parkin*$^{-/-}$/*SOD1*^{G93A} mice, and help pinpoint the pathological stage at which motor deficits occur.

Compared to *parkin*$^{+/-}$/*SOD1*^{G93A} and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates, *parkin*$^{-/-}$/*SOD1*^{G93A} mice maintained their body weight for longer, had delayed hindlimb paralysis, increased lifespan, and less severe motor function deficits on behavioral tests. Tremor onset was not different among the genotypes studied; due to the increased lifespan of *parkin*$^{-/-}$/*SOD1*^{G93A} mice, tremor duration until onset of paralysis as well as overall disease duration were increased in these mice. *SOD1*^{G93A} mice have been reported to show a decline in motor performance on the hang test starting around 80-90 d (Alves et al., 2011), which is also reflected in our data. *Parkin*$^{-/-}$/*SOD1*^{G93A} mice did not begin a steady decline in performance, however, until after 91 d. It is interesting to note that *parkin*$^{+/-}$/*SOD1*^{G93A} mice showed an accelerated decline in motor function as measured by the hang test only, which may be due to the earlier onset of weight loss (early phase of disease) but longer lifespan, thus contributing to increased severity of disease. To more clearly quantify the severity of disease in hindlimb muscle function, future experiments could employ grip strength meters which measure the amount of force an animal applies in grasping pull bar assemblies.

We found that even at end stage, *parkin*$^{-/-}$/*SOD1*^{G93A} mice had less severe symptoms than *parkin*$^{+/-}$/*SOD1*^{G93A} and *parkin*$^{+/+}$/*SOD1*^{G93A} mice. End stage was defined as the point at which mice were no longer able to right themselves after 30 s after being placed on their backs. In the Rotarod test, *parkin*$^{-/-}$/*SOD1*^{G93A} were able to maintain balance on the apparatus for nearly 20 s when the other mice were completely unable to stay on the Rotarod. In addition, the body weight of *parkin*$^{-/-}$/*SOD1*^{G93A} mice at end stage was significantly higher, and these mice lost only 16% of their maximum body weight (compared to about 25% for both *parkin*$^{+/-}$/*SOD1*^{G93A} and *parkin*$^{+/+}$/*SOD1*^{G93A} mice) by

end stage. Though loss of parkin did not completely prevent premature death in *SOD1*^{G93A} mice, our findings demonstrate delayed progression of motor dysfunction and decreased severity of symptoms throughout disease to end stage. The *parkin*$^{-/-}$/*SOD1*^{G93A} mice had a longer total disease duration and longer early phase duration, but the late phase duration was not different among the genotypes studied. Our results indicate that parkin loss successfully prolongs lifespan of *SOD1*^{G93A} mice, specifically in the early phase, which is relevant for translation to human ALS, since treatment in patients is only initiated after disease onset.

The finding that loss of parkin delayed disease progression and prolonged lifespan in *SOD1*^{G93A} mice was surprising, but could be explained by two possibilities. The first possibility is that *in vivo*, parkin may be contributing towards a pathogenic mechanism, so parkin loss is beneficial. Beclin 1 (*BECN1*) is a protein that is involved in autophagosome formation and autophagy activation (Matsunaga et al., 2009; Russell et al., 2013; Zhong et al., 2009). In ALS, mutant SOD1 may abnormally interact with Beclin 1 and cause pathogenic upregulated autophagy; therefore, reducing Beclin 1 in double mutant *Becn1*$^{+/-}$/*SOD1*^{G86R} mice reduced levels of autophagy and increased the lifespan of *SOD1*^{G86R} mice (Nassif et al., 2014). Similarly, parkin has been implicated in promoting autophagy (Olzmann et al., 2007) and any pathogenicity in ALS may be reversed with parkin loss.

The second possibility is that cytoprotective compensatory mechanisms occur under parkin loss. Parkin suppression was recently found to ameliorate behavioral, pathological, and biochemical phenotypes in an AD mouse model with mutated APP (Perucho et al., 2010) and a PD mouse model with mutated α-synuclein (Fournier et al.,

2009), suggesting that parkin loss may be effective in decreasing neurological disease phenotypes due to misfolded, aggregated proteins. In *SOD1*^{G93A} mice, delay in symptom progression and lengthened lifespan are a result of parkin loss, likely due to upregulation of compensatory mechanisms that still need to determined in future studies. Parkin KO mice have been developed by several groups as models of PD (Goldberg et al., 2003; Itier et al., 2003; Perez and Palmiter, 2005; von Coelln et al., 2004), though none have been able to show dopaminergic neuron loss, which is a hallmark of PD pathogenesis. This suggests that knockout of parkin activates compensatory mechanisms that are protective towards dopaminergic neurons. In support of developmental compensation that occurs in germline deletion of parkin, conditional knockout of parkin in adulthood results in dopaminergic neurodegeneration (Shin et al., 2011). This finding indicates that compensatory mechanisms are responsible for the age-dependent activation of dopaminergic degeneration observed, and that parkin deletion since birth masks the neurodegenerative phenotype. Deletion of parkin later in life reveals the neurodegenerative phenotype, which also demonstrates that the lack of phenotype in parkin KO mice with germline mutations is not a human-to-mouse species difference. Therefore, germline mutations that knockout parkin induce mechanisms that protect against dopaminergic degeneration. Our results indicate that loss of parkin also protects against ALS-like motor neuron death in *SOD1*^{G93A} mice. Understanding the underlying mechanisms involved in neuroprotection mediated by parkin loss will be of great therapeutic value to both PD and ALS.

3.4 Materials and Methods

Mice and breeding

Parkin null (*parkin*$^{-/-}$) mice (targeted deletion of exon 2) obtained from Dr. Richard Palmiter (University of Washington) were generated on a coisogenic background (129S4/SvJaeSor) and previously characterized (Perez and Palmiter, 2005). Male transgenic mice overexpressing human *SOD1*^{G93A} (C57BL/6J background) were purchased from Jackson Laboratories (Bar Harbor, ME; stock #00435) and bred with female *parkin*$^{-/-}$ mice. From the resulting F1 generation, male *parkin*$^{+/-}$/*SOD1*^{G93A} mice were bred with female *parkin*$^{+/-}$/*SOD1*$^{+/+}$ mice to generate the F2 generation mice used in experiments, both males and females: *parkin*$^{-/-}$/*SOD1*^{G93A}, *parkin*$^{+/-}$/*SOD1*^{G93A}, and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates. Presence of the human SOD1 transgene (Gurney et al., 1994) and parkin (Perez and Palmiter, 2005) were detected using standard PCR on mouse tail DNA. Genotyping primer sequences were as follows: *SOD1* transgene forward CAT CAG CCC TAA TCC ATC TGA, *SOD1* transgene reverse CGC GAC TAA CAA TCA AAG TGA, internal positive control (mouse *interleukin-2*) forward CTA GGC CAC AGA ATT GAA AGA TCT, and internal positive control reverse GTA GGT GGA AAT TCT AGC ATC ATC C; *parkin* exon 2 forward GAC ATT TCA CTG GCC ATT AAG G, *parkin* exon 2 reverse GAC TCA CTT GAA CCG TCA GGT G, *neo*^{r} forward GAT GTT TCG CTT GGT GGT CGA ATG, and *neo*^{r} reverse CTA TAA AGG GCG TCA CTC AGC CAG. Animals were housed in microisolator cages on a 12-hour light/dark

cycle and given free access to food and water. All animal studies were done in accordance with Emory University IACUC guidelines. The endpoint for mice was defined as when they could no longer right themselves within 30 seconds of being placed on their backs. When hindlimb weakness was observed prior to this endpoint, mice were observed daily for signs of discomfort and given moist chow and HydroGel on the bottom of the cage.

Antibodies

Antibodies used in this study included rhodamine labeled α-bungarotoxin (BTX, Molecular Probes), anti-Protein Gene Product 9.5 (PGP 9.5, Cedarlane), anti-β-actin (Millipore), anti-SOD1 (FL-154, epitope corresponding to full-length human SOD1, Santa Cruz), anti-SOD1 (C4F6, conformational antibody raised against misfolded SOD1 G93A, MediMabs), and anti-parkin (Abcam). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Co-immunoprecipitations and immunoblotting analysis

Total tissue homogenates were prepared from snap frozen mouse brain tissue in 1.1% SDS and lysates were cleared by centrifugation. Equal protein concentrations were subjected to SDS-PAGE and levels of SOD1 and parkin were detected with immunoblotting using anti-β-actin, anti-SOD1, and anti-parkin antibodies. Soluble protein homogenates were prepared from snap frozen mouse brain tissue in lysis buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 1% IGEPAL CA630, and a

proteinase inhibitor cocktail) and lysates were cleared by centrifugation. Protein concentrations in the samples were determined with a BCA Assay kit (Pierce) according to the manufacturer's protocol. Tissue lysates were subjected to immunoprecipitation with anti-parkin antibody, then immobilized on protein G agarose beads. Bound protein complexes were eluted and analyzed using SDS-PAGE followed by immunblotting with anti-β-actin, anti-SOD1, and anti-parkin antibodies.

Behavioral testing

Mice were tested weekly starting at age 5 weeks for their ability to maintain balance on a Rotarod apparatus (Columbus Instruments, OH) with an accelerating paradigm of 1.4 rpm + 4 rpm/min (Fischer et al., 2005). Animals were tested three times during each session, and the longest time before falling off the apparatus was recorded (maximum 600 s). For the hang test, animals were placed on a wire grid, then the grid was flipped upside down on top of an empty plastic cage with a padded bottom (Igoudjil et al., 2011). Latency to falling off the grid was recorded weekly. Mice were also weighed weekly (starting at age 3 weeks) and observed for hindlimb tremor, splaying reflex, and paralysis as seen when lifted up by the tail for a minimum of 10 s (Parone et al., 2013).

End-plate/neuromuscular junction (NMJ) denervation analysis

Tibialis anterior muscles were dissected out from mice that were transcardially perfused with 0.9% NaCl. Muscles were pinned in mild stretch and fixed for 20 min in 4% paraformaldehyde (Sigma) in 1x PBS. Muscles were rinsed in 1x PBS, cryoprotected in

30% sucrose overnight at 4°C, then flash-frozen in supercooled isopentane. Muscles were sectioned at 20 µm and mounted on glass slides. Muscle sections were fixed for 10 min with 4% paraformaldehyde and rinsed with 1x PBS, then stained with rhodamine bungarotoxin (BTX, Molecular Probes) and Protein Gene Product 9.5 (PGP 9.5, Cedarlane) followed by FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). PGP blocking buffer contained 0.3% Triton X-100 and 4% horse serum in 1x PBS. Stained sections were examined with fluorescence microscopy, and every fourth section was counted throughout the entirety of the sectioned muscle (Igoudjil et al., 2011). End-plates were considered innervated if complete overlap with axon terminals was observed, denervated if there was no overlap, and partially innervated if some overlap was observed.

3.5 Figures and Legends

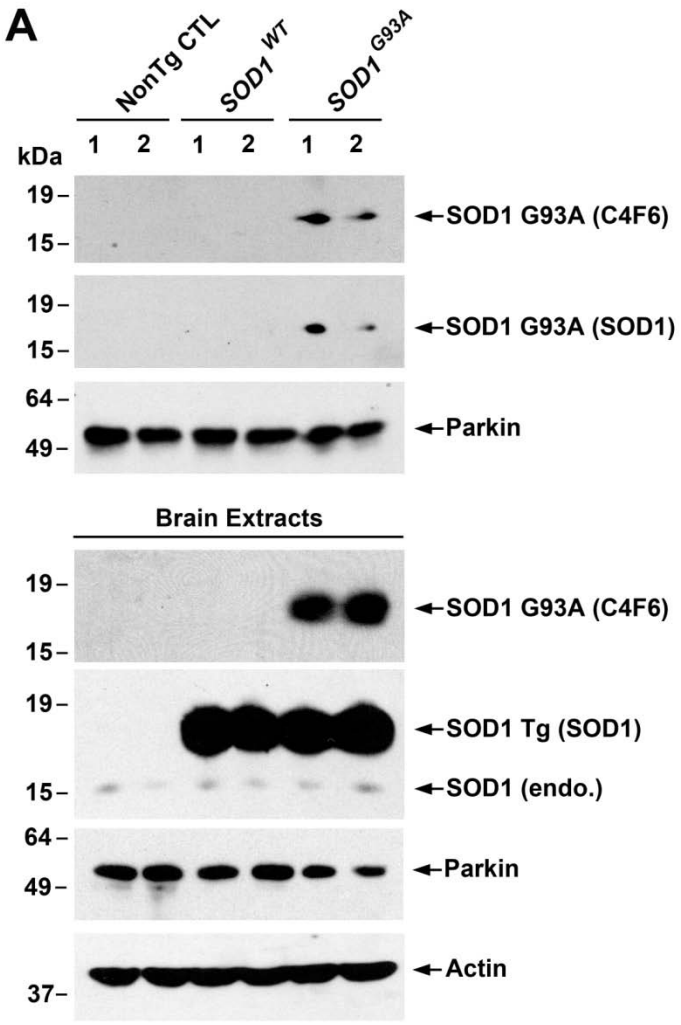

Figure 3.1. Endogenous interaction of parkin and SOD1 G93A. **(A)** Western blot analysis of mouse brain homogenates reveals a specific interaction of parkin and SOD1 G93A. Brain extracts from two individual nontransgenic control (CTL), *SOD1*^{WT} transgenic, or *SOD1*^{G93A} transgenic mice were homogenized in 0.1% Triton X-100 lysis

buffer and subjected to immunoprecipitation with anti-parkin antibody followed by immobilization on protein G agarose beads. Bound protein complexes were eluted from the beads and analyzed with immunoblotting using anti-SOD1 C4F6, anti-SOD1 FL-154, anti-parkin, and anti-β-actin antibodies.

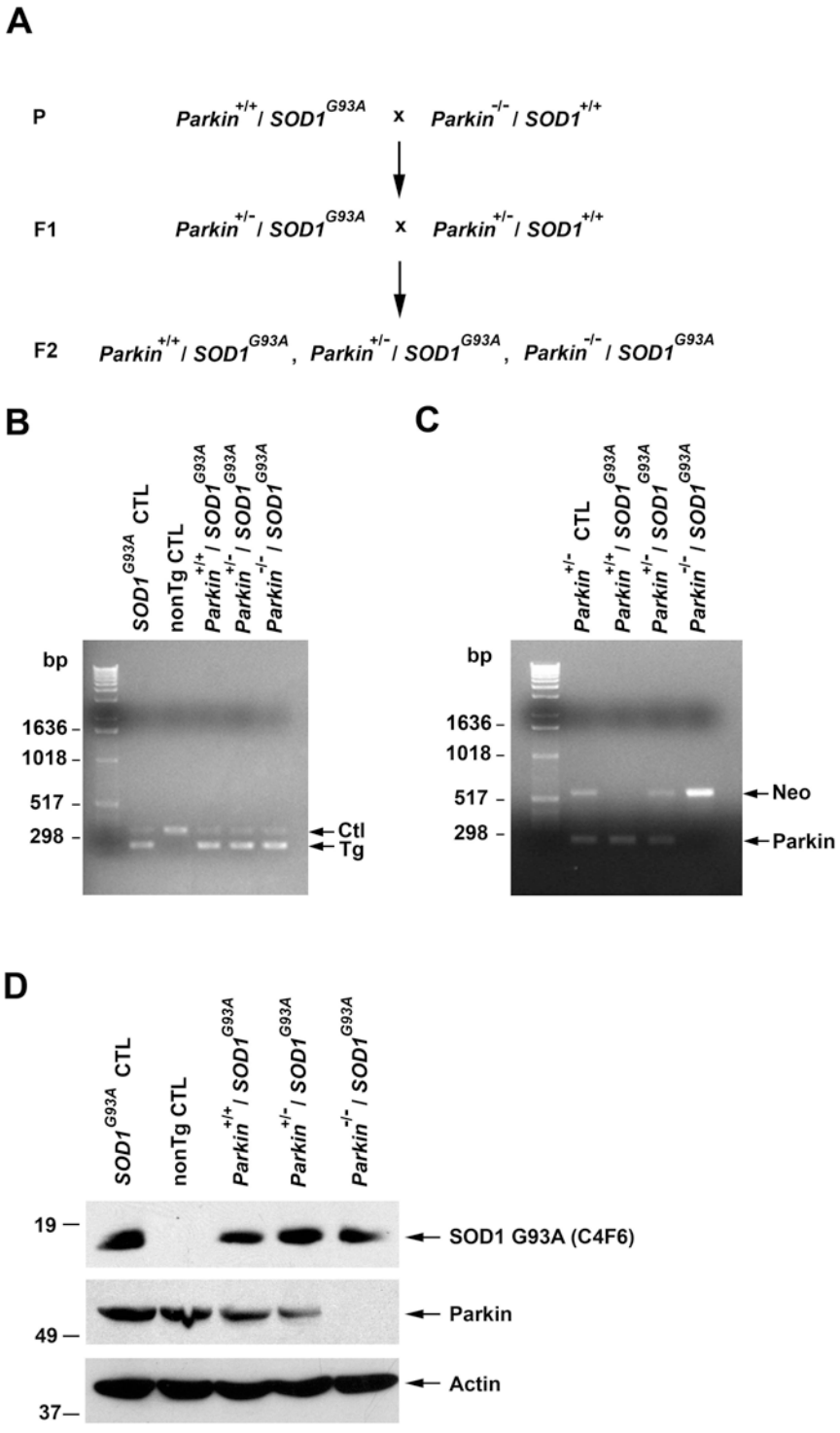

Figure 3.2. Generation and genotyping of double mutant mice. **(A)** Overview of breeding scheme for the generation of *parkin*$^{-/-}$/*SOD1*^{G93A} mice. Male *SOD1*^{G93A} transgenic mice

were bred with female *parkin*$^{-/-}$ mice. From the resulting F1 generation, male *parkin*$^{+/-}$/*SOD1*^{G93A} mice were bred with female *parkin*$^{+/-}$/*SOD1*$^{+/+}$ mice to generate the F2 generation mice used in experiments, both males and females: *parkin*$^{-/-}$/*SOD1*^{G93A}, *parkin*$^{+/-}$/*SOD1*^{G93A}, and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates. **(B)** DNA gel analysis of mouse tail DNA using standard PCR for the transgene (human *SOD1*^{G93A}) and a reference gene (mouse *interleukin-2*) was used to determine progeny that expressed *SOD1*^{G93A}. **(C)** DNA gel analysis of mouse tail DNA using standard PCR for *parkin* exon 2 and *neo*^{r} (replacement vector) was used to determine the parkin genotype of *SOD1*^{G93A} transgenic progeny. **(D)** Western blot analysis of brain homogenates prepared from double mutant mice. Immunblotting using anti-SOD1 C4F6, anti-parkin, and anti-actin antibodies confirmed protein expression of SOD1 G93A and parkin.

Table 3.1. Effect of *parkin* loss on lifespan and disease phenotype

	N	Lifespan (days)	Tremor (days)		Weight Loss (days)		Hindlimb Paralysis (days)	
			Onset	Duration	Onset	Duration	Onset	Duration
***Parkin*$^{+/+}$/ *SOD1*^{G93A}**	17	152.1 ± 2.7	104.7 ± 3.0	36 ± 4.2	106.6 ± 3.7	45.4 ± 2.8	141.0 ± 2.8	11.1 ± 1.6
***Parkin*$^{+/-}$/ *SOD1*^{G93A}**	16	162.1 ± 3.3	108.7 ± 3.0	36 ± 4.4	101.5 ± 5.2	60.6 ± 6.3*	145.1 ± 2.6	16.9 ± 3.1
***Parkin*$^{-/-}$/ *SOD1*^{G93A}**	18	179.1 ± 4.7*	111.2 ± 4.5	56 ± 6.2*	131.4 ± 4.6*	47.7 ± 5.0	164.3 ± 4.1*	15.6 ± 4.6

Table 3.1. Effect of *parkin* loss on lifespan and disease phenotype. Tremor onset was observed by suspending the mouse by the tail and watching hindlimbs for a minimum of 10 s. Duration of tremor was calculated from the onset of tremor to the onset of hindlimb paralysis, when no tremor is observed. Mice were weighed weekly and also at end point, and maximum weight was calculated retroactively to determine weight loss onset. Duration of weight loss was calculated as the time between maximum weight to end point. Hindlimb paralysis onset was observed by suspending the mouse by the tail and watching for loss of the hindlimb splaying reflex. Paralysis duration was calculated as the time between paralysis onset to end point. Values presented are mean ± SEM. * $P <$ 0.05 versus *parkin*$^{+/+}$/*SOD1*^{G93A}, one-way ANOVA with Tukey's post-hoc test.

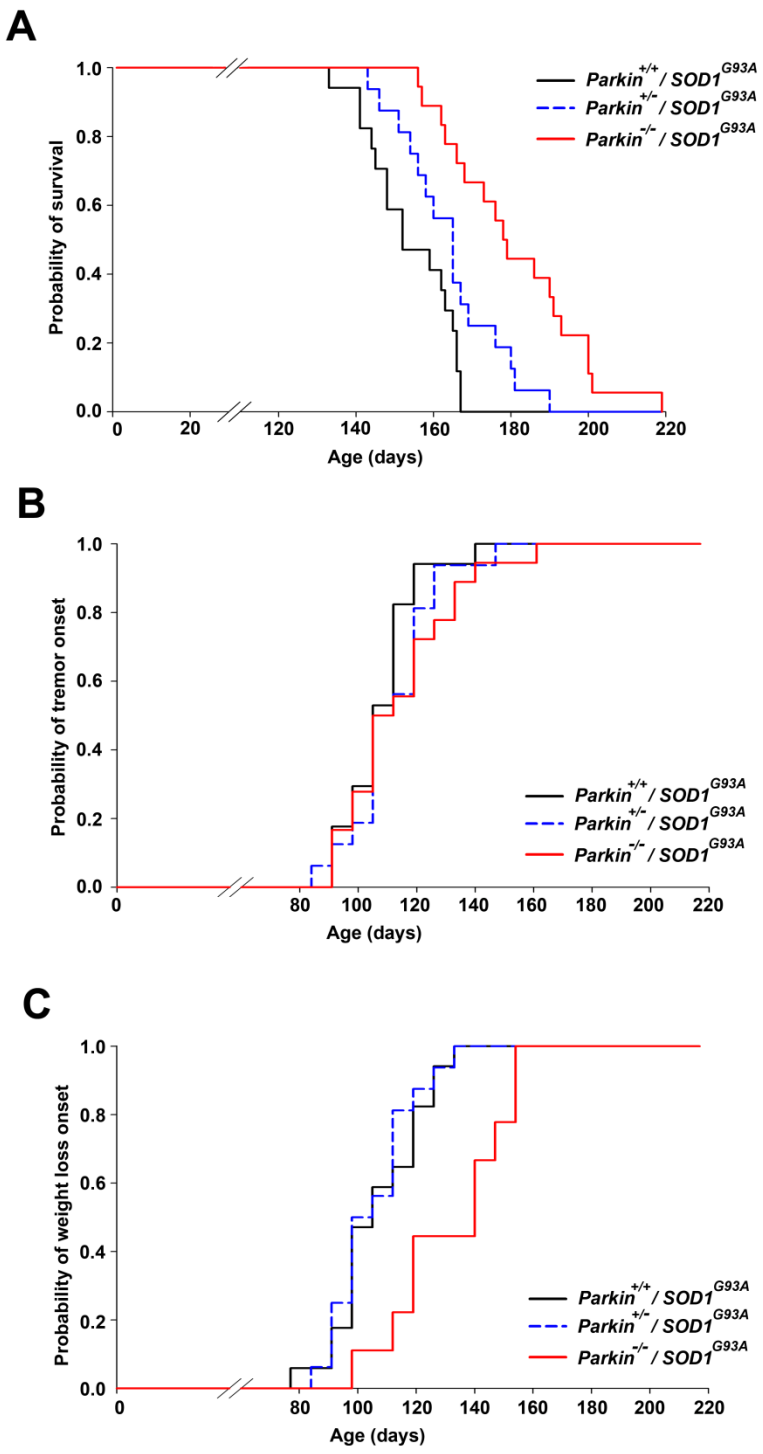

Figure 3.3. Parkin loss extends lifespan without affecting onset of tremor in *SOD1*^{G93A} mice. **(A)** Kaplan-Meier survival analysis of littermates reveal increased survival rate of

parkin$^{-/-}$/*SOD1*^{G93A} mice compared to *parkin*$^{+/-}$/*SOD1*^{G93A} and *parkin*$^{+/+}$/*SOD1*^{G93A} mice. $P < 0.0001$ *parkin*$^{-/-}$/*SOD1*^{G93A} mice versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, log-rank test. $P < 0.0001$ *parkin*$^{+/-}$/*SOD1*^{G93A} mice versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, log-rank test. **(B)** Plotting the probability of hindlimb tremor onset over time shows no difference in the onset of tremor among genotypes. **(C)** Plotting the onset of body weight loss (day at which maximum body weight was reached) over time shows a delay in body weight loss in *parkin*$^{-/-}$/*SOD1*^{G93A} mice. $P < 0.0001$ *parkin*$^{-/-}$/*SOD1*^{G93A} mice versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, log-rank test.

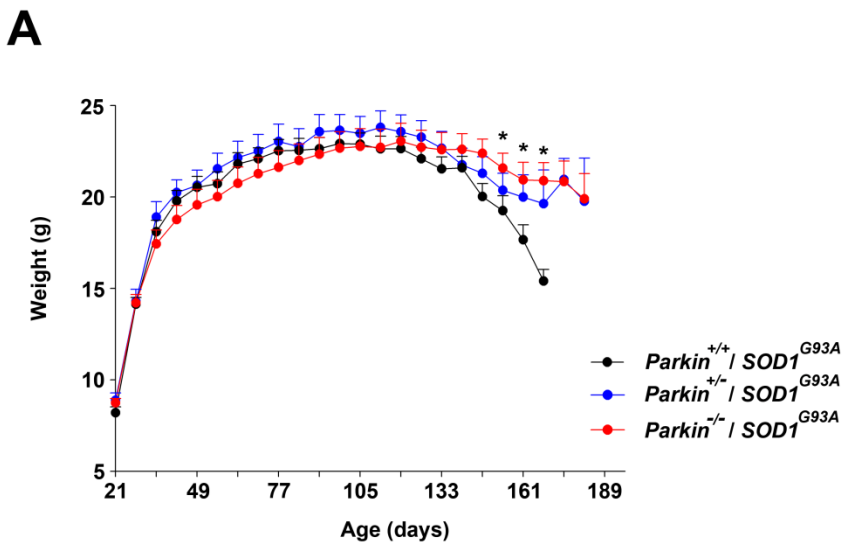

Figure 3.4. Parkin loss delays weight loss in *SOD1*^{G93A} mice. **(A)** Body weight analysis for all surviving animals at each time point shows a significant delay in weight loss for *parkin*$^{-/-}$/*SOD1*^{G93A} mice compared to *parkin*$^{+/+}$/*SOD1*^{G93A} mice starting at age 154 d. * $P < 0.05$ versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, one-way ANOVA with Tukey's post-hoc test.

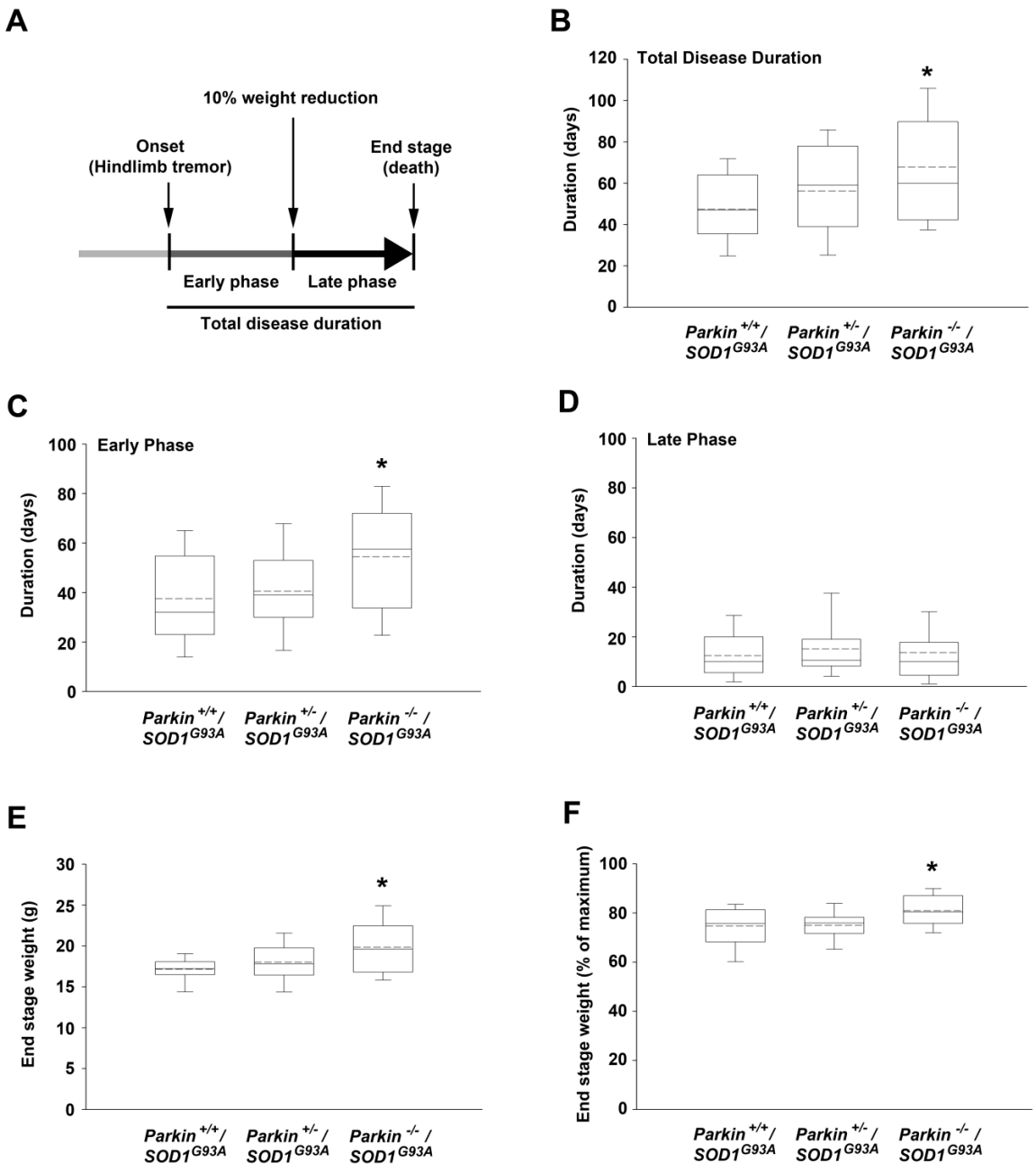

Figure 3.5. Effect of parkin loss on the course of disease progression in *SOD1*^{G93A} mice. **(A)** Schematic diagram defining phases of disease progression. Onset of disease is considered the day at which hindlimb tremors are first observed. The early phase of disease progression consists of the time from tremor onset to a 10% reduction in weight.

The late phase of disease is from the point of 10% weight reduction to end stage (death). **(B-D)** Box plots comparing the range in total disease duration (B), early phase of disease (C), and late phase of disease (D) among *parkin*$^{+/+}$/*SOD1*^{G93A}, *parkin*$^{+/-}$/*SOD1*^{G93A}, and *parkin*$^{-/-}$/*SOD1*^{G93A} mice. **(E)** Box plot of end stage weight in grams shows an overall higher range of body weights in *parkin*$^{-/-}$/*SOD1*^{G93A} mice at end point. **(F)** Box plot of the end stage weights expressed as a percentage of the maximum weight shows that *parkin*$^{-/-}$/*SOD1*^{G93A} mice lost less weight over the course of disease. Dashed lines denote means. * $P < 0.05$ versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice averages, one-way ANOVA with Tukey's post-hoc test.

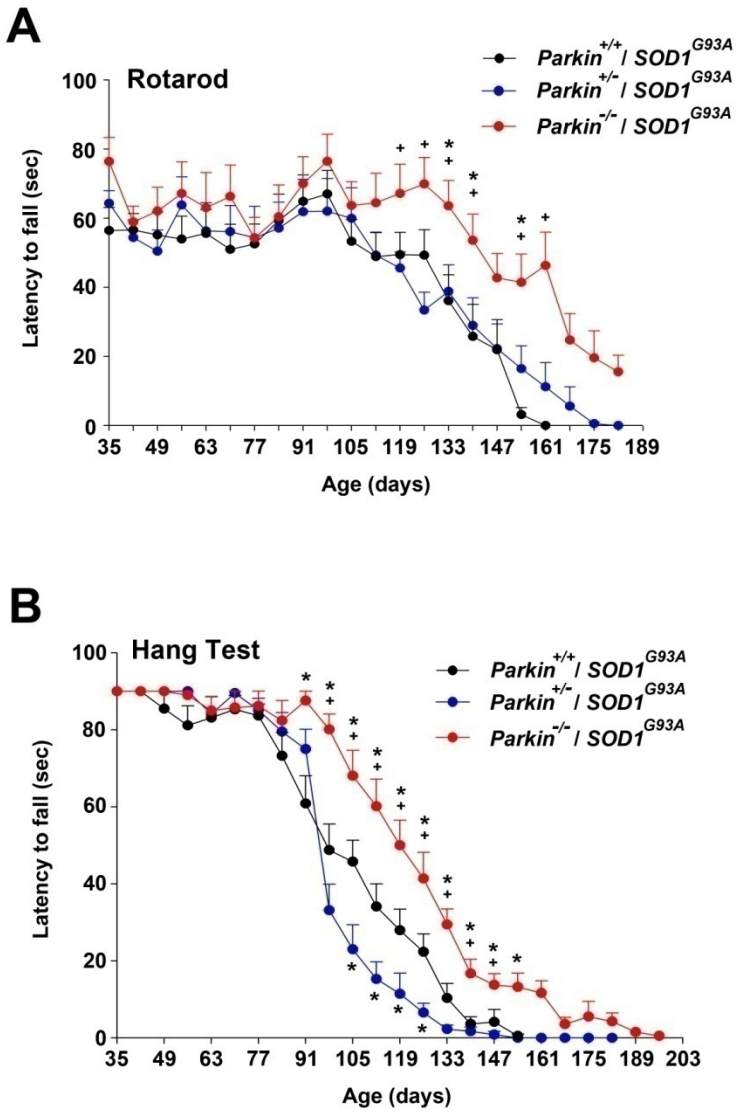

Figure 3.6. Parkin loss ameliorates behavioral phenotype of *SOD1*^{G93A} mice. **(A)** Latency to falling off the Rotarod was recorded for all surviving animals at each time point. Latency to falling was significantly delayed in *parkin*$^{-/-}$/*SOD1*^{G93A} mice compared to *parkin*$^{+/-}$/*SOD1*^{G93A} starting at age 119 d and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates starting at age 133 d. * $P < 0.05$ versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, $^{+}$ $P < 0.05$ versus *parkin*$^{+/-}$/*SOD1*^{G93A} mice, one-way ANOVA with Tukey's post-hoc test. **(B)** Latency to falling off the wire grid in the hang test was recorded for all surviving animals at each time

point. Maintenance of hindlimb grip strength was measured by latency to falling, which was significantly delayed in *parkin*$^{-/-}$/*SOD1*^{G93A} mice compared to *parkin*$^{+/-}$/*SOD1*^{G93A} starting at age 98 d and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates starting at age 91 d. * $P < 0.05$ versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, $^{+}$ $P < 0.05$ versus *parkin*$^{+/-}$/*SOD1*^{G93A} mice, one-way ANOVA with Tukey's post-hoc test. *parkin*$^{+/-}$/*SOD1*^{G93A} showed a significantly accelerated latency to falling compared to *parkin*$^{+/+}$/*SOD1*^{G93A} mice, demonstrating loss of hindlimb grip strength, starting at age 105 d. * $P < 0.05$ versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, one-way ANOVA with Tukey's post-hoc test.

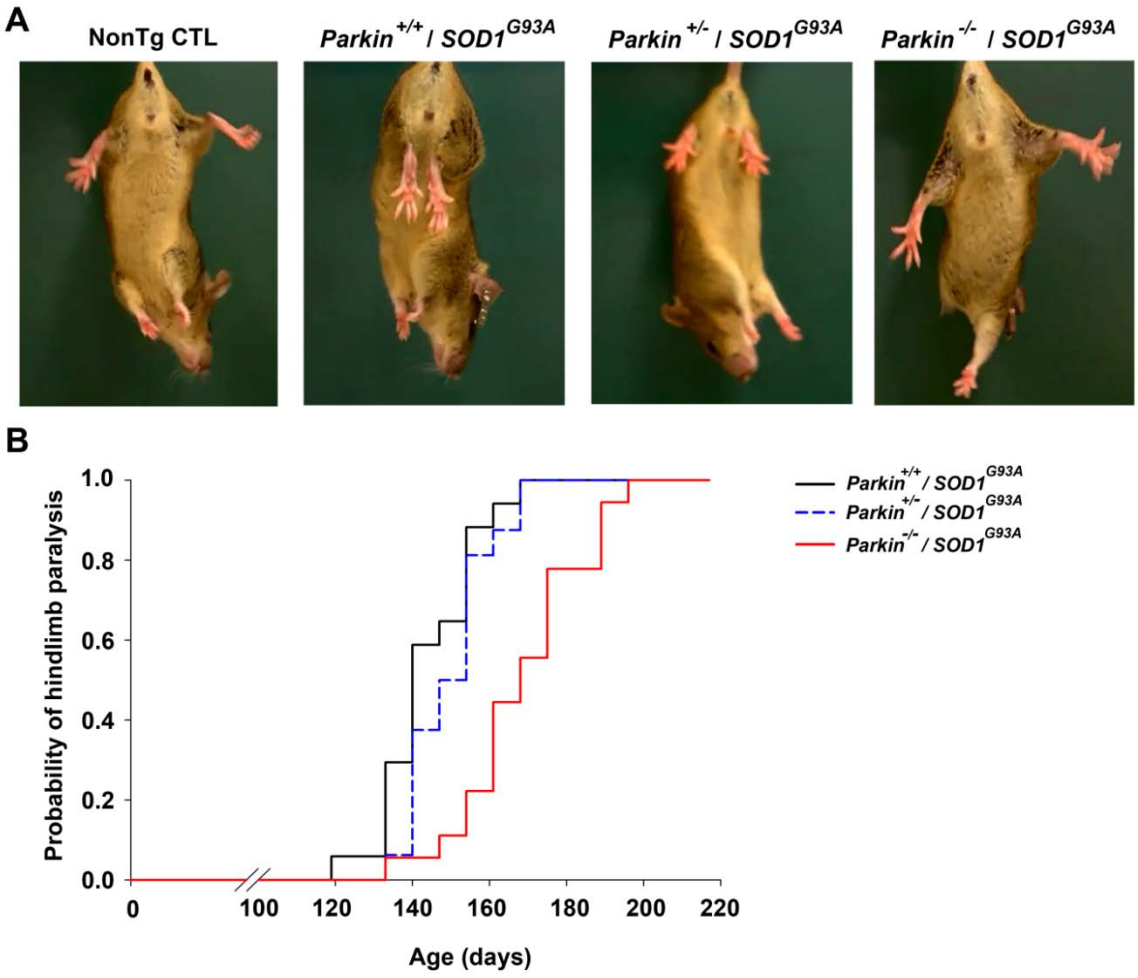

Figure 3.7. Delayed hindlimb paralysis in *parkin*$^{-/-}$/*SOD1*^{G93A} mice. **(A)** When suspended by the tail at age 138 d, nontransgenic CTL mice are able to fully extend their hindlimbs (hindlimb splaying reflex) and retract them without tremor. At the same age, *parkin*$^{+/-}$ /*SOD1*^{G93A} and *parkin*$^{+/+}$/*SOD1*^{G93A} littermates display complete loss of the hindlimb splaying reflex and hindlimbs are mostly restricted to a retracted position typical of later disease progression-related hindlimb paralysis. *Parkin*$^{-/-}$/*SOD1*^{G93A} mice display hindlimb tremor at this age, though no paralysis is observed. **(B)** Plotting the probability

of hindlimb paralysis over time shows delayed paralysis onset in *parkin*$^{-/-}$/*SOD1*^{G93A} mice. $P < 0.0001$ *parkin*$^{-/-}$/*SOD1*^{G93A} mice versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, log-rank test.

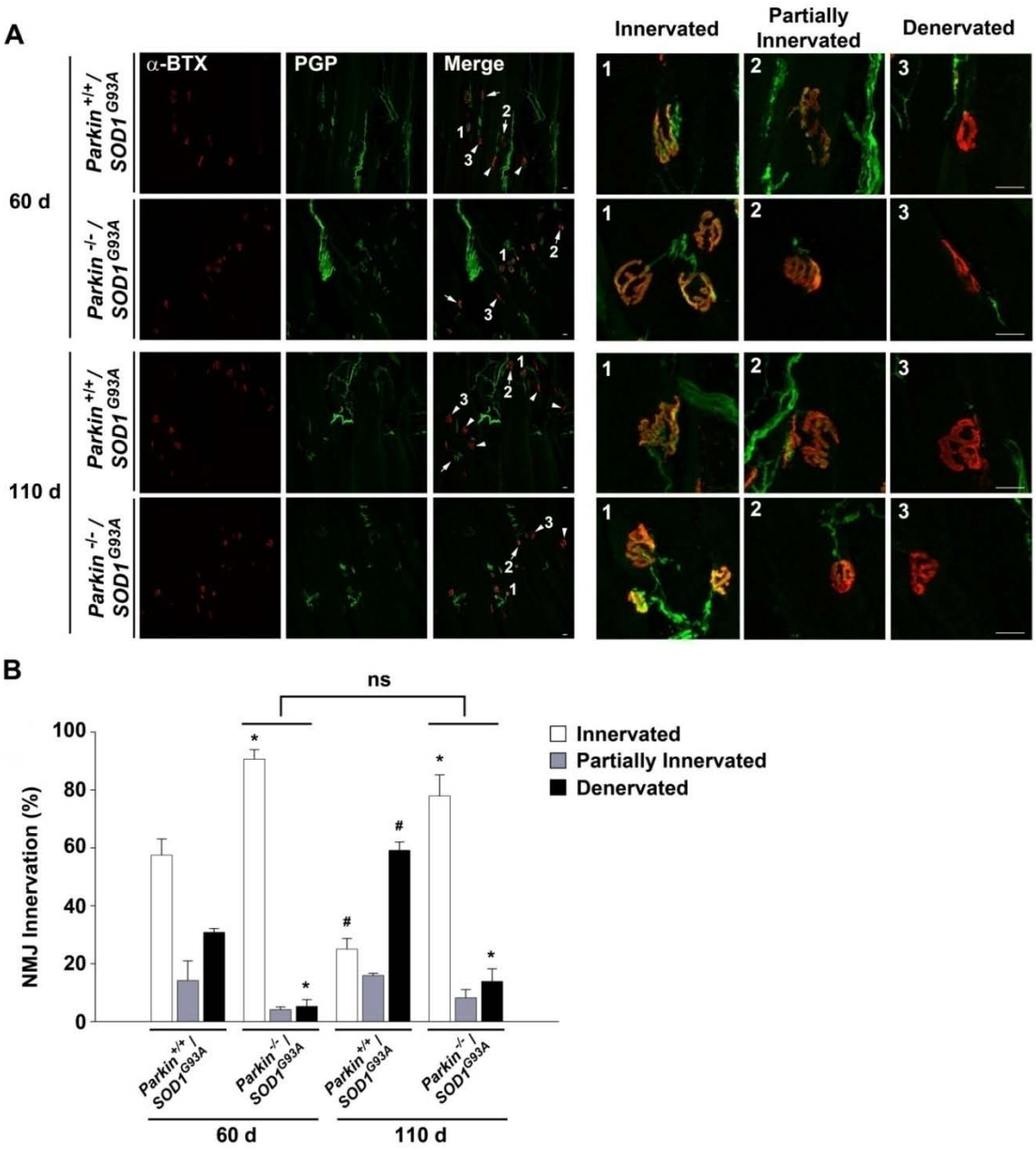

Figure 3.8. Parkin loss decreases denervation of motor endplates in *SOD1*^{G93A} mice. **(A)** Motor endplates at the neuromuscular junction are identified with rhodamine-conjugated α-bungarotoxin which labels post-synaptic acetylcholine receptors (red) and motor neuron axons and terminals are identified with FITC-labeled PGP9.5 (green). Shown are representative images of innervated endplates showing colocalization of motor endplates

and axons (yellow), partially denervated endplates with some colocalization of motor endplates and axons (arrows), and denervated endplates with no colocalization with axons (arrowheads) in *parkin*$^{-/-}$/*SOD1*^{G93A} and *parkin*$^{+/+}$/*SOD1*^{G93A} mice, 60 d and 110 d of age. Insets correspond to the numbered endplates in the merged panels. Scale bar = 20μm. **(B)** Quantitative analysis of the percentage of endplates classified as innervated, partially denervated, or denervated shows decreased endplate denervation for *parkin*$^{-/-}$/*SOD1*^{G93A} at both 60 d and 110 d. * $P < 0.05$ versus *parkin*$^{+/+}$/*SOD1*^{G93A} mice, # $P < 0.05$ versus 60 d old mice, 2-way ANOVA with Tukey's post-hoc test.

Chapter 4: Summary, Implications, and Future Directions

4.1 Summary of findings

Misfolding of SOD1 protein, due to either mutations or to oxidative stress, has been linked to ALS (Andersen, 2006; Hough et al., 2004; Rosen et al., 1993) and causes progressive neurodegeneration of motor neurons (Higgins et al., 2003; Kong and Xu, 1998), indicating an important role for management of misfolded SOD1 protein in cell survival. Disruption of protein quality control mechanisms also contributes to disease pathogenesis (Gestwicki and Garza, 2012), demonstrating the critical role of these processes in cellular health and maintenance. Understanding the molecular mechanisms that regulate degradation of misfolded SOD1 is therefore crucial for elucidating ALS pathogenesis and development of targeted therapeutic intervention.

In Chapter 2, I provided a detailed description of my work regarding parkin-mediated cellular processing of misfolded SOD1 in cells. This novel work is the first to report a specific association of parkin, an E3 ligase known to promote misfolded protein clearance via the aggresome-autophagy pathway, and ALS-linked mutant forms of misfolded SOD1. I found that parkin interacts with misfolded SOD1 G93A and A4V mutants, but not SOD1 WT. Specifically, parkin selectively recognizes misfolded mutant SOD1 and catalyzes K63-linked polyubiquitination on these substrates in cooperation with the UbcH13/Uev1a E2-conjugating complex. Knockdown of parkin resulted in decreased ubiquitination and increased steady-state levels of mutant SOD1, confirming a role for parkin in mediating mutant SOD1 degradation. Parkin overexpression promoted formation of mutant SOD1-containing aggresomes that also contain various aggresome markers such as vimentin, Hsp70, and ubiquitin. These aggresomes were identified as substrates for autophagic clearance by the autophagy-lysosome system as they contained

autophagy markers such as LC3, LAMP2, and p62. Inhibition of autophagy also resulted in accumulation of aggresomes in cells. In addition, I found that parkin is required for proper targeting of misfolded SOD1 to aggresomes, and that parkin mediates cytoprotection against misfolded SOD1 in cells. This work suggests that disruption of parkin-mediated clearance of misfolded SOD1 by the aggresome-autophagy pathway could contribute to ALS pathogenesis.

In Chapter 3, I described the effects of parkin loss in the *SOD1*^{G93A} transgenic mouse model of ALS. Given the cytoprotective effect of parkin against misfolded SOD1-induced toxicity in cells, we expected loss of parkin to exacerbate the ALS-like phenotype found in *SOD1*^{G93A} transgenic mice. Interestingly, loss of parkin resulted in increased lifespan and a significant delay in the progression of disease phenotype in *SOD1*^{G93A} mice. Improved motor function maintenance in *parkin*$^{-/-}$*/SOD1*^{G93A} mice compared to *parkin*$^{+/+}$*/SOD1*^{G93A} or *parkin*$^{+/-}$*/SOD1*^{G93A} double mutant littermates was substantiated with a corresponding delay in NMJ denervation, even at an early symptomatic stage of disease. *Parkin*$^{+/+}$*/SOD1*^{G93A} mice, on the other hand, showed an age-dependent increase in NMJ denervation. Under conditions of parkin loss, compensatory mechanisms may be activated and delay disease progression in *SOD1*^{G93A} mice.

4.2 Implications of parkin function in ALS

This dissertation work has demonstrated, for the first time, a novel interaction between the E3 ligase parkin and ALS-linked mutant SOD1. Parkin recognizes misfolded SOD1 mutant protein and promotes its formation into aggresomes and subsequent clearance by autophagy. These findings demonstrate a role for parkin-mediated autophagy in misfolded SOD1 protein clearance.

Parkin-mediated cytoprotection via autophagy

There is some evidence that autophagosome formation is not impaired, but actually upregulated, in several neurodegenerative diseases, suggesting that cells may increase autophagy in an attempt to prevent accumulation of misfolded proteins and remove protein aggregates. For example, autophagy-related genes are transcriptionally upregulated in AD patient brains (Lipinski et al., 2010), and ALS patients are reported to have increased levels of Atg5-Atg12 and autophagosomes (Hetz et al., 2009; Sasaki, 2011). Additionally, there are reports that in motor neurons from ALS mouse models and ALS patients, the UPS is impaired while a compensatory pathway of autophagy is upregulated (Li et al., 2008; Sasaki, 2011). In support of this notion, induction of autophagy via a complex of HspB8/Bag3/Hsc70/CHIP decreased aggregation and slowed ALS-like disease progression in *SOD1*^{G93A} mice (Crippa et al., 2010b).

On the other hand, there is growing evidence for autophagic dysfunction in ALS, and defects in autophagic flux may contribute to motor neuron degeneration (Banerjee et al., 2010). Treating *SOD1*^{G93A} mice with the autophagy inducer rapamycin caused

accumulation of autophagic vesicles, but failed to reduce the level of mutant SOD1 aggregates in the spinal cord, suggesting underlying defects in clearance of autophagic vesicles through lysosome fusion and possible abnormal autophagic flux in ALS (Zhang et al., 2011). Rapamycin treatment also caused accumulation of p62 (Zhang et al., 2011), which is normally degraded during autophagy, in support of impaired autophagosome clearance in *SOD1*^{G93A} mice. Excess autophagic activity may actually lead to autophagic stress and subsequent neurodegeneration (Cherra and Chu, 2008; Chu, 2006). It is important to note that increased autophagic vacuoles do not necessarily correlate with upregulated autophagy. Instead, when accumulated autophagic vacuoles are observed in degenerating neurons, there may be an imbalance between autophagic sequestration of cargo and completion of the degradation pathway, which can be cytotoxic (Chu, 2006). Mutations in valosin-containing protein (VCP), a recently identified risk factor for ALS, lead to impaired autophagosome-lysosome fusion (Ju et al., 2009), further demonstrating autophagic dysregulation in ALS. While autophagosome formation was initially increased in *SOD1*^{G93A} mouse muscle, induction of autophagy by starvation resulted in reduced autophagy in *SOD1*^{G93A} mice instead of enhancing autophagy like in control mice (Xiao et al., 2015), further supporting a role for abnormal autophagy in ALS. In addition, mutant SOD1 protein itself can inhibit autophagic machinery by causing mislocalization of dynein/dynactin and impairing clearance (Zhang et al., 2007). Taken together, these findings suggest that impaired autophagy contributes to ALS pathogenesis.

The cytoprotective effect of parkin overexpression against misfolded SOD1 A4V and G93A found in Chapter 2 is at odds with the cytoprotective effect of parkin loss

against SOD1 G93A found in Chapter 3. However, I believe these differences are due to the different model systems used and compensatory mechanisms that may be activated in mice that were not present in our cell culture model. In SH-SY5Y cell culture, studies were performed with transient overexpression of parkin and mutant SOD1. Furthermore, aggregation and clearance of mutant SOD1 was assessed within a few hours or days after transfection whereas in mice, we studied the effects of parkin loss throughout the entire lifespan of the organism. In these mice, mutant SOD1 is expressed and parkin expression is lost during embryogenesis and throughout their development and lifespan. Across a lifespan and stages of development in a whole mouse model, we hypothesize that other cellular pathways could compensate for the loss of parkin and provide cytoprotection against misfolded SOD1 via a yet unknown alternate pathway (discussed further in section 4.3).

Regulation of SOD1 by parkin in neurodegenerative diseases

Increased levels of total SOD1 protein were significantly increased in both AD and PD patient brains compared to age-matched controls, raising the possibility that oxidative modification of wild-type SOD1 in these samples could induce misfolding (Ezzi et al., 2007) and/or protease resistance of SOD1, promoting accumulation and aggregation (via decreased clearance) of SOD1 in cells (Choi et al., 2005). SOD1-immunoreactive protein aggregates were also observed in AD brains (Choi et al., 2005). In cells where parkin expression is knocked down using shRNA, we found an increase in total levels of mutant SOD1, suggesting that loss of parkin function could contribute to disease pathogenesis by preventing clearance of misfolded SOD1. Future studies are needed to determine if these pathogenic parkin mutants alter ubiquitination and clearance

of misfolded SOD1, and whether altered parkin-SOD1 binding is a factor for inclusions found in PD.

We have shown that parkin is important for inclusion body formation, recruiting misfolded DJ-1 L166P (Olzmann and Chin, 2008; Olzmann et al., 2007) and SOD1 mutants to aggresomes. Loss-of-function mutations in parkin may contribute to defective degradation pathways and cause early-onset PD, which has been shown to be almost entirely devoid of Lewy bodies (Farrer et al., 2001; Mori et al., 1998; Takahashi et al., 1994), supporting our hypothesis that parkin is needed for aggresome and inclusion body formation. Previous to the work detailed in this dissertation, there had not been specific evidence of mutant SOD1 recruitment to aggresomes by parkin. SOD1 is frequently reported as a component of Lewy body-like hyaline inclusions found in ALS patients, and SOD1 immunoreactivity was also reported in Lewy bodies from PD patients (Nishiyama et al., 1995), suggesting that a common pathological process involving accumulated SOD1 is responsible for the formation of Lewy bodies and other neuronal intracytoplasmic inclusions. In addition, a case study of an fALS patient with a slowly progressing form of the disease showed no abnormalities in his SOD1 cDNA sequences, as well as a lack of Lewy-body-like hyaline inclusions (Kato et al., 1996), which demonstrates a possible role for mutant SOD1 in inclusion formation. Whether or not parkin was involved in these reports is unknown; however, future studies on parkin-mediated aggregation and clearance of mutant or misfolded, oxidatively damaged SOD1 and subsequent neuroprotection in PD could better inform whether this mechanism extends to neurodegenerative diseases beyond ALS.

4.3 Future Directions

What signaling mechanism(s) trigger parkin-mediated SOD1 ubiquitination?

In parkin's compact, normally autoinhibited state (Trempe et al., 2013; Wauer and Komander, 2013), the zinc-binding RING0 domain blocks the active site cysteine of RING2 and a significant conformational rearrangement would need to occur for the active site to be unmasked and bring RING1 (bound to the E2-Ub complex) to the RING2 active site (Berndsen and Wolberger, 2014). While the structural details of parkin activation are largely unknown, PINK1-mediated phosphorylation on Ser-65 of parkin (Kazlauskaite et al., 2014) has been described as the primary method of parkin activation, especially for mitophagy. When mitochondria are damaged, PINK1 phosphorylates parkin and recruits it to the depolarized mitochondria, where parkin ubiquitinates outer mitochondrial membrane proteins to promote mitophagy (Narendra et al., 2008). Our lab has previously shown that PINK1 phosphorylation results in parkin activation and K63-polyubiquitination of the parkin substrate IKKγ by parkin (Sha et al., 2010). These studies demonstrate the versatility of PINK1-mediated parkin activation towards diverse protein substrates. Whether PINK1 also contributes to parkin-mediated polyubiquitination of misfolded SOD1 is unknown.

On the other hand, parkin may specifically ubiquitinate different substrates as a result of different posttranslational modification signals. Mutant SOD1 is reported to undergo a number of posttranslational modifications, including cysteinylation, glutathionylation (Redler et al., 2011), and palmitoylation (Antinone et al., 2013; Auclair et al., 2013; Redler et al., 2011). It is unknown whether any posttranslational

modifications on misfolded SOD1 are responsible for parkin recruitment to trigger ubiquitination and autophagy.

To test whether parkin is activated by PINK1 phosphorylation prior to ubiquitinating mutant SOD1, SOD1 ubiquitination assays could be performed using overexpression of PINK1 WT or kinase-dead PINK1 mutants. If PINK1 phosphorylation is required for parkin-mediated ubiquitination of mutant SOD1, one would expect PINK1 WT overexpression to increase subsequent SOD1 ubiquitination compared to baseline endogenous levels of PINK1 or kinase-dead PINK1. PINK1 null mice have been developed as a model of early onset PD due to PD-linked PINK1 loss of function mutations, though these mice do not display dopaminergic neurodegeneration (Gispert et al., 2009; Kitada et al., 2007; Zhou et al., 2007). Experiments can be done to study exogenously expressed mutant SOD1 ubiquitination and mutant SOD1 levels in cells cultured from these mice compared to WT controls. If PINK1 is required for parkin's activation and ubiquitination of mutant SOD1, one would expect the loss of PINK1 to decrease ubiquitination of mutant SOD1, resulting in accumulation of mutant SOD1 protein. To test whether various posttranslational modifications of mutant SOD1 enhance binding to parkin or SOD1 ubiquitination, *in vitro* cysteinylation, oxidation, palmitoylation, or other modifications could be performed followed by *in vitro* ubiquitination assays. Modified mutant SOD1 could then be subjected to *in vitro* binding assays with parkin to determine whether any modification enhances the interaction with parkin.

On a larger scale, other SOD1 binding partners could be screened in order to study other potential modulators of mutant SOD1 regulation, perhaps via yet unidentified

posttranslational modifications. Immunoprecipitation of misfolded SOD1 from transgenic mice or ALS patient samples could recover other SOD1 interactors, which would be identified by mass spectrometry. Confirmation of their interaction would be performed by *in vitro* or cell-based *in vivo* co-immunoprecipitation and colocalization studies. Any posttranslational modifications on mutant SOD1 could be determined by western blot or mass spectrometry, and further experiments can be done to probe the functional effects of the modification, such as testing for altered aggregation, localization, or clearance from cells. Cysteine residues in human SOD1 are most commonly modified, particularly Cys-6 and Cys-111 (Antinone et al., 2013; Auclair et al., 2013; Redler et al., 2011), and could be candidate modification sites. In $SOD1^{H46R}$ transgenic mice which bear the mutated form of human SOD1, peroxidation of Cys-111 promoted the formation of insoluble, aggregated SOD1 that correlated with the progression of motor dysfunction. However, in $SOD1^{H46R/C111S}$ mice, where Cys-111 is replaced with Serine and peroxidation of Cys-111 is subsequently blocked, motor neuron disease onset occurred later and disease progression slowed compared to $SOD1^{H46R}$ mice (Nagano et al., 2015). These findings show that Cys-111 is a critical amino acid residue for modulating toxicity of mutant SOD1. The two remaining cysteine residues, Cys-57 and Cys-146, are unlikely to be modified since they form a stable intramolecular disulfide bond (Abernethy et al., 1974). Interestingly, the DUB Ataxin-3 was shown to edit K63-polyubiquitin chains on mutant SOD1 and promote aggresome formation (Wang et al., 2012), raising the possibility for further protein interactions even following parkin-mediated ubiquitination.

What are the compensatory mechanisms activated by parkin loss in SOD1^{G93A} mice?

In *SOD1^{G93A}* mice, parkin loss resulted in a delay in symptom progression and lengthened lifepsan, likely due to activation of compensatory mechanisms that still need to determined in future studies. Parkin KO mice have been developed by several groups as models of PD (Goldberg et al., 2003; Itier et al., 2003; Perez and Palmiter, 2005; von Coelln et al., 2004); however, none have been able to show dopaminergic neuron loss, which is a hallmark of PD pathogenesis. This suggests that germline knockout of parkin activates compensatory mechanisms that are protective towards dopaminergic neurons. In support of developmental compensation that occurs in germline deletion of parkin, conditional knockout of parkin in adulthood results in dopaminergic neurodegeneration (Shin et al., 2011). This finding indicates that compensatory mechanisms are responsible for the age-dependent dopaminergic degeneration observed, and that parkin deletion since birth masks the neurodegenerative phenotype. Deletion later in life reveals the neurodegenerative phenotype, which also demonstrates that the lack of phenotype in parkin KO mice with germline mutations is not a human-to-mouse species difference. Studies using shRNA to knock down parkin have shown an acute effect that could differ from germline deletion of parkin. For example, acute knockdown of parkin in mouse livers using shRNA reduced mitophagy, exacerbating acetaminophen-induced liver injury (Williams et al., 2015). However, genomic deletion of parkin modeled in parkin KO mice showed that mitophagy still occurred in the absence of parkin, and parkin KO mice were protected against acetaminophen-induced liver injury compared to control mice (Williams et al., 2015). Therefore, germline mutations that knockout parkin induce mechanisms that protect against cytotoxic effects such as dopaminergic degeneration.

Our results also indicate that germline loss of parkin protects against ALS-like motor neuron death in *SOD1*^{G93A} mice. Current studies in ALS model mice utilizing a variety of therapeutic strategies, including drug treatments, recombinant protein injections, vaccination, and cross-breeding, have all shown quite mild increases in lifespan ranging from about 9 to 20 days (see Table 1.3 in Chapter 1 for summary). Similarly, Riluzole only prolongs life by about 3 months in human ALS patients, underscoring the urgent need for better therapeutic strategies. The experiments described in Chapter 3 show a significantly increased lifespan in *SOD1*^{G93A} mice with homozygous deletion of parkin, at about 27 days, which suggests that parkin modulation could be an improved therapeutic target.

Germline parkin loss may trigger multiple pathways of compensatory cell protection in *SOD1*^{G93A} mice. To determine if parkin is critical for motor neuron protection and the presence of compensatory mechanisms in ALS mice, *SOD1*^{G93A} mice could be studied using a conditional parkin knockout. One approach would be to use a floxed mouse strain with the loxP-flanked parkin (usually a selected exon of parkin) and cross it with a *SOD1*^{G93A} mouse strain harboring the inducible Cre recombinase gene (such as Cre-ERT2, a fusion protein of Cre and a mutant estrogen receptor ligand-binding domain). Crossing the two strains would provide an inducible conditional knockout when the activity of Cre-ERT2 is induced by administration of tamoxifen in adulthood. A more direct approach would be knockout of parkin early in postnatal development (before disease onset) in *SOD1*^{G93A} mice by stereotactic injection of lentiviral GFP-Cre recombinase in *SOD1*^{G93A} mice with parkin exon (such as exon 7 (Parone et al., 2013)) flanked by loxP sites. Loss of parkin, specifically in spinal cord and brain, could be

confirmed by immunohistochemistry and immunoblotting. If ALS-like disease progression and motor neuron loss occurs following parkin deletion, this would indicate that parkin is important for cytoprotection against misfolded SOD1-induced toxicity, which would reconcile with the data presented in cells from Chapter 2. Mice with induced conditional knockout of parkin, as well as cells, would not have the compensatory mechanisms in place that occur from germline parkin deletion in the traditional parkin KO mice. Therefore, this type of study would demonstrate whether germline parkin KO-induced compensatory mechanisms are cytoprotective.

Another explanation for the longer-lived *parkin*$^{-/-}$/*SOD1*^{G93A} mice could be that ubiquitination of SOD1 G93A increases its toxicity in mice. In the case of α-synuclein in PD, proteasome impairment causes an increase in levels of the E3 ligase Siah-1, which ubiquitinates α-synuclein in a degradation-independent manner and promotes α-synuclein aggregation and toxicity in cells (Lee et al., 2008). Depletion of Siah-1 resulted in decreased α-synuclein ubiquitination and rescued cytotoxicity induced by α-synuclein under proteasomal impairment (Lee et al., 2008). It is unknown whether any E3s promote this type of degradation-independent ubiquitination and aggregation of misfolded SOD1. To address this question, future studies in *parkin*$^{-/-}$/*SOD1*^{G93A} mice would need to evaluate the levels of ubiquitinated SOD1 G93A found in the spinal cord and brain of mice and determine whether loss of parkin affects degradation and steady-state levels of mutant SOD1. Based on the Siah-1 and α-synuclein model, one might expect that in mice, yet unidentified compensatory mechanisms cause parkin-mediated ubiquitination of SOD1 G93A to aggregate and cause further toxicity in mice. Future studies would need to also determine whether parkin promotes degradation of misfolded

SOD1 in mice, since another E3 that ubiquitinates misfolded SOD1 in a degradation-independent manner would need to be identified.

Alternatively, the interaction of parkin with mutant SOD1 could be deleterious in *SOD1*^{G93A} mice, so loss of parkin would counter this toxicity. It was recently reported that *SOD1*^{G86R} mice with heterozygous loss of Beclin 1 have increased lifespan (Nassif et al., 2014). Beclin 1 is a protein involved in nucleation and expansion of autophagosomes in the autophagy pathway (Kihara et al., 2001; Matsunaga et al., 2009; Zhong et al., 2009). The authors hypothesize that ALS model mice have abnormal levels of autophagy due to abnormal association of mutant SOD1 and Beclin 1; therefore, reduction of Beclin 1 levels in *Becn1*$^{+/-}$*/SOD1*^{G86R} mice attenuated the pathological effects (Nassif et al., 2014). In addition, deletion of HDAC6 (a ubiquitin-dynein adaptor protein involved in autophagy) in *SOD1*^{G93A} mice extended survival in these mice, which was associated with increased levels of acetylated α-tubulin (Taes et al., 2013). Increased acetylation of α-tubulin facilitates axonal transport via recruitment of kinesin-1 and dynein (Dompierre et al., 2007; Reed et al., 2006). This report suggests that modulation of axonal transport in the absence of HDAC6 is beneficial in the ALS-like disease state. Taken together with our findings, it is possible that the benefits of deletion of certain proteins involved in autophagy outweigh the potential harmful effects of their loss, especially if the disease state or expression of mutant SOD1 causes deleterious autophagy regulation or toxicity.

Loss of parkin in a *SOD1*^{G93A} mouse model leads to increased lifespan and delays symptom progression. This finding may prompt one to speculate whether parkin inhibition could be a therapeutic strategy in ALS. No parkin-specific pharmacological antagonists have been identified to our knowledge; however, a number of E3 ligase

inhibitors are commercially available. These compounds are currently mostly studied in preclinical development for anti-cancer therapies (Bielskiene et al., 2015; Skaar et al., 2014). F-box proteins, a component of SCF (Skp1-Cullin-F-box) family ubiquitin ligases, have been implicated in both driving and opposing cell proliferation. Therefore, nonspecific inhibition of SCF E3 ligases could be problematic due to interfering with both cellular processes. However, some SCF complexes have been well-characterized, which could drive the advancement of rational drug development (Skaar et al., 2014), though SCF-family specific inhibitors may not affect parkin activity, since parkin is a member of the RBR E3 ligase family. Some molecular mechanisms of inhibiting parkin ubiquitination have been described. SUMOylation of parkin, for example, has been shown to reduce parkin substrate ubiquitination in favor of parkin autoubiquitination, resulting in parkin translocation to the nucleus (Eckermann, 2013; Um and Chung, 2006). The DUB ubiquitin-specific protease 15 (USP15) was recently shown to counteract the parkin-mediated ubiquitination of outer mitochondrial membrane proteins in mitophagy (Cornelissen et al., 2014). Much more research will need to be performed in the double-mutant mouse model to validate this approach, given that it is more likely that germline deletion of parkin upregulates other compensatory mechanisms that are protective for motor neurons (discussed previously), rather than simply studying postnatal antagonism of parkin in regards to cytoprotection.

Hypotheses for the compensatory upregulation of certain proteins in response to germline loss of parkin focus primarily on chaperones and antioxidants. In an AD mouse model, parkin deficiency resulted in increased levels of chaperone proteins (Perucho et al., 2010), and in a PD mouse model, parkin deficiency was hypothesized to increase

levels of reduced glutathione (Fournier et al., 2009), both leading to improved disease phenotype. Indeed, a proteomics study in *parkin* KO mice, which do not display PD-like dopaminergic neuron degeneration, detailed the altered abundance of proteins that may be involved with adaptive mechanisms and neuroprotection in the *parkin* KO mice (Periquet et al., 2005). In particular, proteins involved in stress response, protein degradation, and detoxification were increased in *parkin* KO mice.

Parkin loss may trigger multiple pathways of cell protection in $SOD1^{G93A}$ mice, most notably, an increase in molecular chaperone activity. Recent studies comparing two $SOD1^{G93A}$ mouse strains with similar mutant SOD1 expression levels and ALS-like phenotype but on different genetic backgrounds revealed some underlying differences contributing to a rapidly progressive disease (129Sv background) versus a slowly progressing one (C57B6 background) (Marino et al., 2015; Nardo et al., 2013). Elevated levels of functional chaperones such as cyclophillin-A and chaperone alpha-B-crystallin (CRYAB) were features associated with the slow progressing pathology in $SOD1^{G93A}$ mouse models (Marino et al., 2015); therefore, increased chaperone activity could have underlying benefits in a $SOD1^{G93A}$ ALS model.

Activation of the heat shock pathway and upregulation of Hsp70 expression by administration of the small molecule Hsp90 inhibitor BIIB021 ameliorated proteasome dysfunction in fibroblasts from a patient with peripheral myelin protein 22 (PMP22) duplication-associated Charcot-Marie-Tooth type 1A (Chittoor-Vinod et al., 2015). This study also showed that Hsp70 is critical for preventing PMP22 aggregation under proteotoxic stress, and that Hsp70 contributes to removal of misfolded PMP22 by autophagy, although the detailed mechanism is not yet understood. To highlight the

importance of chaperone activity in PQC, recent studies suggest that chemical chaperones, small molecules designed to have higher activity levels than molecular chaperones, are of interest in treating neurodegenerative diseases by preventing misfolding and aggregation (Cortez and Sim, 2014). In fact, a new chemical chaperone amide derivative was shown to improve the neurological function and delay weight loss in an ALS mouse model (Getter et al., 2015). Additionally, overexpression of the Hsp40 chaperone family member HSJ1 in *SOD1*^{G93A} mice reduced misfolded SOD1 aggregation and enhanced motor neuron survival (Novoselov et al., 2013). Taken together, these data suggest that under conditions of parkin loss and proteasomal inhibition, an alternative pathway of elevated Hsp70 or other chaperones can be activated in a last-resort attempt to clear misfolded SOD1 from cells.

Oxidative stress is a prime contributor to ALS pathogenesis. SOD1 is a major antioxidant protein and fALS cases caused by mutant SOD1 are thought to have aberrant antioxidant activity (Bruijn et al., 2004; Simpson et al., 2003). In studies of cerebrospinal fluid and blood samples from ALS patients, several markers of free radical damage have been identified (Turner et al., 2009), suggesting an imbalance in antioxidant activity in ALS. Indeed, oral administration of MitoQ, a mitochondria-specific antioxidant drug (Tauskela, 2007) to *SOD1*^{G93A} mice was able to decrease the severity of disease, specifically in improving mitochondrial function (Miquel et al., 2014), demonstrating that oxidative stress in mitochondria plays a role in ALS pathogenesis. Parkin null mice are reported to have elevated levels of reduced glutathione in the brain (Casarejos et al., 2009), which promotes resistance to epoxomicin-induced cell death. Overexpression of genes involved in glutathione biosynthesis has also been shown to be cytoprotective in a

Drosophila model of synucleinopathy (Trinh et al., 2008). Together, these findings suggest that elevated levels of the antioxidant reduced glutathione could be beneficial for cytoprotection. Testing the double-mutant mice generated in our studies for levels of reduced glutathione using glutathione peroxidase colorimetric assays could provide insight into whether parkin knockout in these mice also have increased levels of reduced glutathione. Further analyses of oxidative stress in these mice would determine whether compensatory increases in reduced glutathione could rescue the oxidative stress due to mutant SOD1.

Could parkin-mediated autophagy also result in delayed disease progression in mice?

Given the role of parkin overexpression described in Chapter 2 and the findings of others, we hypothesize that autophagic clearance is an important cytoprotective mechanism against mutant SOD1-induced toxicity (Crippa et al., 2013; Han et al., 2015; Zhang et al., 2014). Misfolded SOD1-containing inclusions may interfere with several cellular functions, such as axonal transport, mitochondrial function, and PQC/protein degradation activities, contributing to motor neuron death (Cozzolino et al., 2008; Pasinelli and Brown, 2006; Seetharaman et al., 2009). Therefore, clearance of misfolded SOD1 may be beneficial for alleviating ALS symptoms. Vaccination of low-copy $SOD1^{G93A}$ mice with recombinant SOD1 G93A or apo-SOD1 WT (non-metallated, misfolded SOD1 WT) resulted in generation of anti-SOD1 G93A antibodies, potentiated protective immunity (increased interleukin-4 and reduced IFNγ), delayed symptom onset, and increased lifespan of $SOD1^{G93A}$ mice (Takeuchi et al., 2010). Vaccination of $SOD1^{G37R}$ and $SOD1^{G93A}$ mice with recombinant SOD1 G93A also delayed disease onset

and extended lifespan (Urushitani et al., 2007). In this study, western blot analysis using C4F6, a monoclonal antibody generated against misfolded SOD1 G93A, showed a decrease in misfolded SOD1 burden in the vaccinated *SOD1*^{G37R} mice, indicating that clearance of misfolded SOD1 helped alleviate disease symptoms in *SOD1*^{G37R} mice. Though the method of misfolded SOD1 clearance was not explored in this study, it is apparent that clearance of misfolded SOD1 is central in cytoprotection. Overexpression of parkin in a mouse model of AD with mutant APP was able to rescue synaptic plasticity and behavioral symptoms, in addition to decreasing β-amyloid protein load and APP protein levels (Hong et al., 2014), suggesting that parkin mediates clearance of misfolded proteins and is therefore a potential target for treatment of neurodegenerative disease.

The finding that overexpression of the E3 ligase dorfin in *SOD1*^{G93A} mice decreased the severity of symptoms as well as motor neuron degeneration (Sone et al., 2010) provides additional evidence that E3-mediated clearance of misfolded SOD1 is beneficial in an ALS model. Dorfin, an RBR-type E3, is reported to specifically recognize misfolded SOD1 and reduce SOD1-induced cellular toxicity, as well as mediate proteasomal clearance of misfolded SOD1 in cells (Niwa et al., 2002). Misfolded SOD1 is reported to undergo both autophagic and proteasomal degradation (Kabuta et al., 2006); however, the Dorfin study in cells did not explore the possibility of autophagic degradation, instead solely testing proteasome inhibition using MG132 treatment. Misfolded proteins found in diseased tissue often make up aggregates that are too large to undergo proteasomal degradation (Bence et al., 2001; Bennett et al., 2005), which indicates the need for a bulk-degradation process such as the aggresome-autophagy clearance system in these instances. *SOD1*^{G93A} transgenic mice show

accumulation of SOD1-positive inclusions, increased levels of autophagy markers, and impaired UPS function (Cheroni et al., 2009; Cheroni et al., 2005; Morimoto et al., 2007). Taken together, it is likely that parkin E3 ligase activity may mediate autophagic degradation of misfolded SOD1 and confer cytoprotection in an ALS mouse model.

Gene therapy is a relatively new field that uses the delivery of genes into cells to treat disease, including ALS (Federici and Boulis, 2012). Traditional drug-based approaches may alleviate symptoms, but do not correct the underlying genetic problems. FALS linked to SOD1 is caused through a toxic gain-of-function; therefore, RNA interference (RNAi) has been proposed to knockdown mutant SOD1 expression. However, attempts to silence the SOD1 mutant gene have shown mixed results for modulating ALS disease progression. Lentiviral vector-mediated gene silencing of *SOD1* in $SOD1^{G93A}$ mouse hindlimb muscles led to retrograde transport of the vector and >50% transduction efficiency in the spinal cord ventral horn, increasing the lifespan of $SOD1^{G93A}$ mice (Ralph et al., 2005). However, systemic (intravenous) delivery of recombinant adeno-associated virus (rAAV6) shRNAs for *SOD1* resulted in efficient body-wide transduction of rAAV6-shSOD1 in all skeletal muscles, but only low levels of transduction in motor neurons, and did not prevent ALS-like disease progression in $SOD1^{G93A}$ mice (Towne et al., 2008). In this case, the level of gene silencing achieved may not have been sufficient to combat SOD1-induced cytotoxicity, particularly if there were low levels of transduction in motor neurons. An alternative therapy, with a much higher reported success rate, is to target the overexpression of cytoprotective factors to ALS-affected tissues. There has been success in motor neuron protection and slowed disease progression demonstrated with viral vector-mediated delivery of neurotrophic

factors GDNF, IGF-1, and VEGF in transgenic SOD1 mutant mice (Acsadi et al., 2002; Azzouz et al., 2004; Kaspar et al., 2003). In addition, gene delivery of anti-apoptotic proteins Bcl-xL and Bcl-2 protected motor neurons *in vitro* and in SOD1 mutant mice (Azzouz et al., 2000; Garrity-Moses et al., 2005; Yamashita et al., 2002).

Viral delivery of parkin has proven beneficial in preclinical studies modeling PD (Coune et al., 2012; Mochizuki, 2009; Mochizuki et al., 2008), but this approach has not yet been well-described in ALS models. Parkin interacts with glycosylated α-synuclein and ubiquitinates it in normal human brain; therefore, it is hypothesized that loss of parkin in AR PD causes accumulation of α-synuclein and PD pathogenesis (Shimura et al., 2001). Overexpression of α-synuclein in the substantia nigra of monkeys and rats has been shown to induce dopaminergic cell death (Kirik et al., 2003; Klein et al., 2002; Yamada et al., 2004). A PD rat model expressing rAAV2-α-synuclein and also transduced with rAAV2-parkin showed less cell toxicity and dopaminergic neuron loss due to α-synuclein (Yamada et al., 2005), suggesting that parkin gene therapy is effective against α-synucleinopathy. Furthermore, lentiviral delivery of WT parkin to A30P-α-synuclein transgenic rats resulted in reduced α-synuclein-induced neuropathy and increased survival of dopaminergic neurons (Lo Bianco et al., 2004). One recent study in a rat model of ALS overexpressing TDP-43 demonstrated that stereotaxic injection of lentiviral parkin into the primary motor cortex rescued neurons from TDP-43-induced apoptosis (Hebron et al., 2014). Though this study did not examine lifespan and motor function in the rats or the molecular consequences of parkin overexpression regarding clearance of TDP-43, it points to the benefits of parkin overexpression in an ALS model. More studies will need to be conducted to further elucidate the mechanisms of

cytoprotection due to viral parkin overexpression in neurodegenerative disease models, but initial data suggests a beneficial effect in reducing misfolded protein load.

Both lentiviral and AAV vectors are able to achieve robust expression levels, but AAV is a more translational approach in the long term since it is currently the only viral vector approved for use in human clinical trials. In addition, AAV is able to achieve long-lived gene expression without a deleterious inflammatory response, which makes it ideal for expression in spinal cord, which is sensitive to inflammation (Snyder et al., 2011). Directly targeting the tissue of interest in ALS by intrathecal administration of AAV6 or AAV9 have been shown to allow the widest distribution of cell transduction throughout the spinal cord, particularly when expressed under the CMV promoter (Snyder et al., 2011). Serotypes differ in their natural tropisms for a particular tissue and/or cell type. AAV6 may be a good choice for retrograde transport to motor neurons via intramuscular injection (Towne et al., 2009; Towne et al., 2010), while AAV9 has been demonstrated to penetrate the blood-brain barrier after intravenous administration, which could be optimal for transduction of CNS tissues (Duque et al., 2009; Foust et al., 2009). A future experiment to test the effects of directly targeting parkin overexpression to the spinal cord in *SOD1*^{G93A} mice could involve intrathecal administration of AAV9-parkin. The level of transduction would first be confirmed by immunohistochemical analyses of spinal cord tissue. Levels of autophagy markers and clearance of SOD1-containing aggregates could also be observed by immunohistochemistry to determine if parkin overexpression mediates autophagic clearance of misfolded SOD1. Next, motor neuron innervation would be evaluated by methods similar to those described in Chapter 3. Finally, longitudinal studies would be conducted by recording ALS-like symptom

onset and lifespans, as well as evaluating motor function using behavioral tests. If overexpression of parkin directly in the spinal cord is successful in ameliorating disease via autophagy, one would expect fewer aggresomes in the spinal cord, increased innervation of motor neuron endplates, and delayed symptom onset and death in the transduced *SOD1*^{G93A} mice compared to controls.

Markers for autophagy are often increased in the spinal cord motor neurons of ALS model *SOD1*^{G93A} mice, including LC3-II and p62, as well as increased numbers of autophagic vacuoles (Morimoto et al., 2007; Zhang et al., 2011). Autophagy activation is thought to be protective in some neurodegenerative diseases by removing cytotoxic proteins (Berger et al., 2006; Pan et al., 2008; Spilman et al., 2010). However, evidence for the deleterious effects of autophagy overactivation suggests that a balance in misfolded SOD1 clearance is critical for cellular health. Rapamycin is commonly used to induce autophagy by inhibiting mammalian target of rapamycin (mTOR). Rapamycin administration to *SOD1*^{G93A} mice accelerated motor neuron degeneration and shortened lifespan with no obvious effects on SOD1 aggregate accumulation despite elevated levels of autophagy markers (Zhang et al., 2011). In this case, rapamycin exacerbated the pathology of ALS model mice by activating apoptosis (Zhang et al., 2011). Therefore, fine-tuning the amount of autophagy induced as well as considering other pathways that may be altered due to modulation of autophagy will be critical in elucidating therapeutic avenues for ALS.

4.4 Hypothesized model of parkin in regulation of misfolded SOD1

Many questions are still unanswered regarding the effects of modulating parkin level on clearance of misfolded SOD1. However, based on my dissertation work and established findings, a potential model of parkin-mediated cytoprotection against misfolded SOD1 can be proposed. Under proteasomal impairment, misfolded SOD1 is normally cleared from cells through parkin-mediated K63-polyubiquitination and subsequent degradation via the aggresome-autophagy pathway, which we found to be cytoprotective with parkin overexpression. When parkin expression is knocked down, parkin-mediated clearance of misfolded SOD1 is conversely decreased, which could lead to cytotoxicity due to misfolded SOD1. On the other hand, compensatory mechanisms are likely activated in *parkin*$^{-/-}$*/SOD1*^{G93A} mice, which results in delayed disease progression and increased lifespan. Hypotheses for compensatory pathways include increased levels of molecular chaperones, the antioxidant reduced glutathione, and increased autophagy via an alternate E3 ligase. Further studies of mechanisms that regulate the clearance of misfolded SOD1 in both cell and animal models will aid our understanding of ALS pathogenesis and reveal insights into the role of parkin in neurodegenerative diseases.

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